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Egr1 transcription factor is an immediate early gene that is induced in response to many stimuli including stimuli that cause genotoxic stress and DNA damage. Egr1 plays a key role in stress responses that follow chemotherapy or irradiation treatments for breast cancer, by stimulating apoptosis. In addition, Egr1 and p53 both play roles in cell cycle arrest and DNA repair stress responses that can allow the cell to repair DNA damage before proliferating. We developed a protocol to determine which genes are regulated by Egr1 during these activities by subjecting cells to irradiation and a technique called chromatin immunoprecipitation (ChIP). We collect the promoter sequences that were bound to Egr1 protein at the time of fixation, process the DNA to amplify and then label with fluorescent dyes for hybridization on a microarray of identified promoter DNAs. Scanning and special software that normalizes and calculates the signal to control ratios followed by bioinformatic analyses indicates the regulated genes. Many genes important to breast cancer were revealed such as PTEN, p53 and the GADD45 which are all are transactivated by Egr1. With further work we can determine if any new genes are targets for cancer therapy.

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Cloning Egr-1 Regulated genes in breast cells

INTRODUCTION

We have made good progress on both Task 1 and Task 2 of the Statement of work. We have applied the chromatin crosslinking and immunoprecipitation (ChIP) method to MCF7 breast cancer cells and to MCF10A normal breast cells and other cell types when appropriate, for comparison. For example, prostate cancer cells are particularly suitable for Egr1 target identification with ChIP because Egr1 is constitutively over-expressed in these cells.

One refinement is the making and use of a promoter array that we have made in collaboration of two laboratories at the neighboring Sidney Kimmel Cancer Center. With this tool it is not necessary to make cDNA libraries for cloning new genes. However, one new gene called TOE1 (target of Egr1-1) was cloned using that method. The protein product of TOE1 has been largely characterized, and a publication is attached. To replace the labor-intensive cDNA library route we have developed a microarray in collaboration with two neighboring laboratories.

A microarray allows a highthroughput method to identify hundreds of target genes simultaneously, we have results showing cell-type specific sets of genes that are regulated by Egr1 in different conditions. The most striking genes that we have identified and focused on are PTEN, p53 (both tumor suppressor genes that are mutated or inactivated in 50% of tumors), and GADD45 family of genes that specify DNA repair after genotoxic stress stimulation of cells. Many other new genes have yet to be individually examined for therapeutic applications.

SCIENTIFIC BODY

SOW-1

We have used all the sub task components a) to e) (except b and c) listed in the SOW to make three major contributions to the list of genes that play roles in breast cancer:

As reported last year, we have cloned a new gene, named TOE1 for "Target of Egr1-1" from HT 1080 fibrosarcoma cells. This work was done by Dr Ian de Belle in the laboratory, who is partially funded by a postdoctoral Fellowship from the DOD BCRP. This work has now been developed into a published paper (de Belle et al., 2003), whose content will be reported by Dr de Belle. He continues to work on this project.

SOW-2

We have used the methods described in subtasks a) to d) and achieved the retrieval of a list of genes that are still being assessed for possible roles in aspects of stress response and apoptosis or survival. The large number of genes recovered is going to take much longer to characterize for possible marker gene potential.

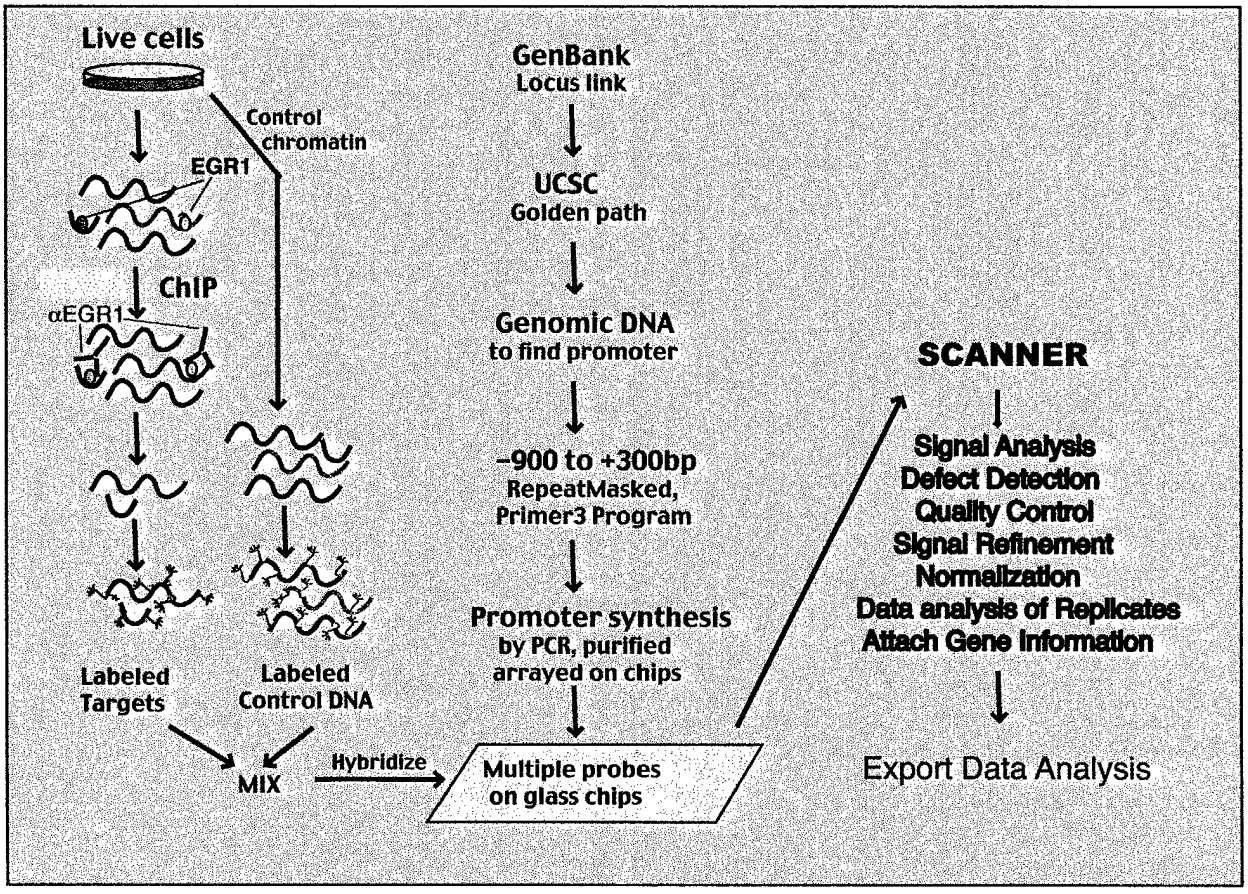
We reported last year that one important target gene regulated by Egr1 is PTEN (Virolle et al., 2001). We reported our findings in Nature cell biology, and a copy is attached in the Appendix. It was not known at that time how PTEN was regulated. We were the second

group to report a transcriptional regulator for PTEN because a different transcription factor, p53, was also shown to up-regulate PTEN transcriptionally [Stambolic, 2001 #7249]. It turns out that the situation is much more complex, because although both p53 and Egr1 individually upregulate the transcription of PTEN, thay are additive (unpublished data). Moreover, Egr1 can upregulate p53 transcriptionally (Ahmed, 2004; Nair et al., 1997), and these two proteins can interact physically(Liu et al., 2001). Furthermore, both proteins can bind the co-activator pair of proteins known as p300/CBP (unpublished findings of J Yu and ED Adamson). We have discovered that this pair of genes is also transcriptionally regulated by Egr1. This was the work of Dr Jianxiu Yu in my laboratory who was awarded a PCRP post-doctoral Fellowship for his research application on this topic and he will be describing his findings in a separate report.

The promoter microarray

This was made with funds from other sources and currently has ~5000 DNA samples spotted onto glass chips in triplicate on each slide. This is done at the SKCC Genomic core facilities, in collaboration with Dr Michael McClelland. The overall process is summarized in Figure 1 below.

Fig.1 The overall process of ChIP on a chip

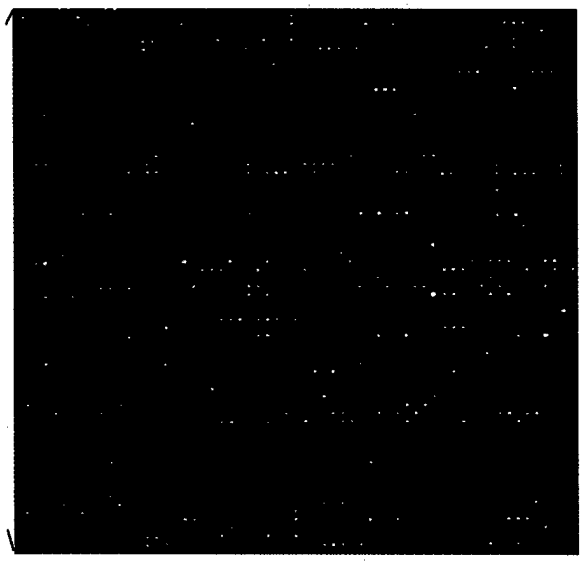


As shown In Figure 1 the array of identified promoters was made on the assumption that most regulatory domains of genes lie between —1000 and +500, relative to the start of

transcription. Therefore, we selected primers for PCR that would encompass this region of the selected genes in order to make double-stranded DNA that would contain this region, on average, the DNA is 1200 bp long. We chose genes that have been associated with cancer, tumor suppressors, oncogenes, transcription factors, cell cycle, stress response, DNA repair, growth arrest, senescence, differentiation, etc, by preference. We chose only those whose sequences have been confirmed and annotated. The dye-labeled ChIP products are hybridized to the array and scanned by ScanArray and QuantArray software.

A portion of a typical array after staining is shown in **Figure 2** after it has been hybridized to fluorescently-labeled DNA from ChIP and after it has been washed and scanned. Each spot represents one promoter DNA.

Figure 2. Section of promoter array hybridized with Egr1 ChIP DNA fragments captured from breast cancer cells (red). The control was genomic DNA (green),



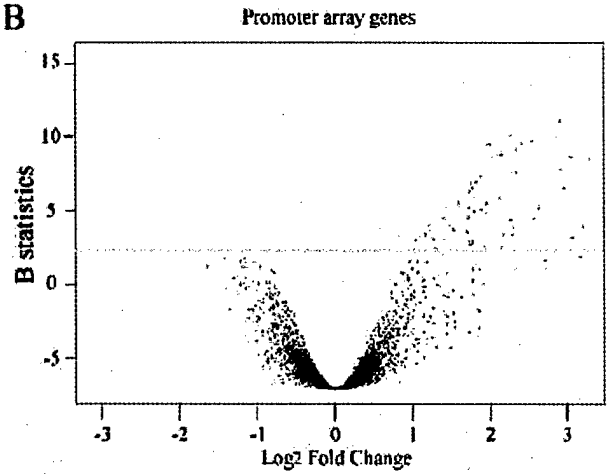


Fig. 3 Volcano plot of the same data with the spot data on the upper RHS representing the target genes to 95% confidence limits

The Analysis of the signals by complex bioinformatics techniques and programs such as the R analysis allows the signal of the reactive genes to be recognized above the noise of the control DNA. The analysis results in the sort of data seen in **Figure 3**, as a volcano plot where each spot in the upper right quandrant represents a gene that is bound by Egr1 with 95% confidence.

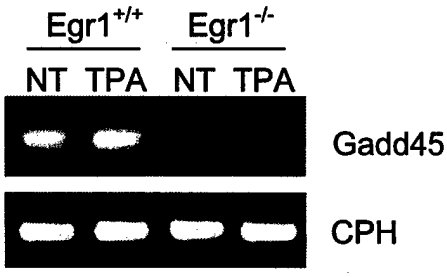
Every gene identified in this way will have to be verified as genuine Egr1 target genes in a number of tests. Before starting the characterizing of new genes, we use a battery of tests to verify that the new putative target genes that are found by their signals on the microarray, really are targets. For instance we measure whether the expression of the new gene increases or decreases in parallel with Egr1 during induction. This is best done with QRT-PCR to measure the mRNA levels. Figure 4 is a table where a sample of typical results

indicate that when Egr1 increases after tumor promoter treatment (TPA) the levels of Akt2 and Pim1 also increase. The induction level is different in breast (MCF7) cells compared to that in prostate cancer (DU145) cells.

Figure 4. Validation of genes as targets of Egr1
QRT-PCR was used to assay the induction of Egr1 and 2 putative Target genes after induction of Egr1 with tumor promoter TPA.

	DU145		MCF7	
	NT	TPA	NT	TPA
Egr1	1.0	130.4	1.0	100.2
Akt2	1.0	19.6	1.0	1.4
Pim1	1.0	2.0	1.0	17.7

Figure 5. Egr1 is required for Gadd45A induction (Cyclophilin Control).



In **Figure 5** we show the verifyication that Egr1 is required for the regulation of a gene, by the use of the Egr1 null mouse embryo fibroblast (MEF) compared with the wild-type cells. We show here that the Egr1-/- MEF expresses Gadd45A mRNA very poorly in untreated (NT) and very little better in TPA-induced cells, in contrast to wt Egr1 MEFs where GADD45A is expressed in untreated cells and this increases in TPA —treated cells. This confirms that Egr1 is an important stimulator of stress response genes expecially when p53 is absent or mutant as in these cells, because p53 is also a regulator of this gene.

In **Figure 6** we make use of the mouse embryo finbroblas(MEF)t knock out cellsagain to show that the GADD45A gene is a likely direct target of regulation b Egr1

Figure 6. Egr1-/- MEFs cannot respond to Egr1 By elevation of GADD45A gene

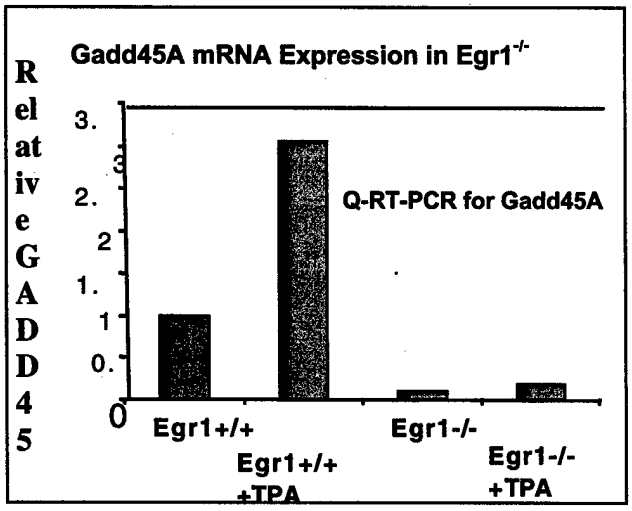
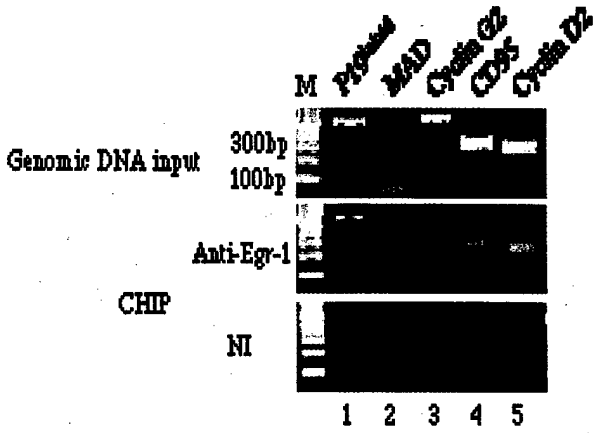


Figure 7. Four new Egr1 target genes shown after cross-linking and PCR



We have also used Affymetrix analyses to confirm the direction of the effect of target genes after stimulation or after antisense Egr1 treatment of cancer cells (Virolle et al., 2003). This method reveals the parallel changes in expression of thousands of genes when Egr1 levels are changed. This is how we determined the identity of several possible new targets of Egr1 such as INK4d cell cycle inhibitor, MAD, Cyclin D2 and CD95 but not Cyclin G2.

The verification of 4 of these 5 possible target genes was demonstrated as follows. If these are direct target genes, Egr1 should bind to the expected site in the promoters of these genes and can be tested with "conventional ChIP" (**Figure 7)** in which ChIP-captured DNA is the template for PCR using primers specific for a short piece of the promoter of the test gene. The predicted DNA band is amplified by PCR to confirm the presence of the promoter in the captured DNA population. Non-immune DNA (NI) is the negative control and input DNA is the positive control.

The data that we have accumulated is currently being analyzed and validated for publication. A number of cluster analyses are shown in the **Appendix** section. For example, we demonstrate that the target genes of Egr1 identified in Serum-stimulated MCF7 and MCF10A cells is quite different from those in prostate cells and differs according to the stimulus type. In addition, we show that the cluster of genes bound is specific for Egr1 target genes, and for Sp1 and P53 regulated genes.

We have formed collaborations with colleagues who were interested in finding out the mechanism of the activation of PTEN by IGF2, the growth factor, in breast cells. We were able to show that IGF2 induces Egr1 and this induces PTEN and this was published (Moorehead and Edwards DR, 2003) and a copy is attached.

KEY RESEARCH ACCOMPLISHMENTS

1. The identifcation of a new gene that is highly expressed in most cells at high density and causes the cells to arrest at G2. This is called TOE1.
2. The publication of two review papers on Egr1 in cancer (attached)
3. The improvement of the idententification of Egr1 target genes using an "in house" promoter array, because there is no commercial source.
4. The promoter array was prented at the Era of Hope Conference, Orlando Fa, sept 2002.
5. The collection of a long list of genes that may reveal useful cancer markers after further analyses.
6. The identification and verification of GADD45A, CD95, Cyclin D2, MAD, p19/Ink4d as target genes is important as they are largely concerned in signaling towards apoptosis or DNA repair after genotoxic stress.

REPORTABLE OUTCOMES

1. Seven publications have been produced with all or partial support of this DOD award.
2. I attended the Era of Hope Conference and gave a talk on "Egr1 target genes in breast cancer detected using promoter arrays".
3. As a result of the work on the PTEN gene, the collaboration with Dr Tomas Mustelin in this Institute was taken further to develop the hypothesis that Egr family of proteins

may be important in PTEN and its effect on T cell function. An RO1 grant from the NIH was awarded last year with T Mustelin and PI.

4. A DOD PCRP Fellowship award was made to Dr Jianxiu Yu to work on the transcriptional regulation of p300/CBP pair of related genes
5. I was invited to present data on the promoter array at two academic Institutes, one in San Antonio Institute of Biotechnology, Texas, April, 2003, one in Naples Italy, Oct, 2003, at the Genetics Institute and a third to the Cambridge Health-Research Institute (CHI) organized "PEPTALK" Conference in San Diego in Jan 2004.
6. Several collaborations have been set up to use our method of highthroughput technology to determine the promoters that are regulated by other transcription factors in addition to Egr1. Dr Mercola at SKCC is writing a paper on the effect of cis-platin on breast cancer cells and the genes that are regulated by c-Jun and ATF2. Several other groups have received slides (gratis) and are currently using them.

CONCLUSIONS

Egr1 is an important transcription factor with the experimentally advantageous feature that it is markedly up-regulated by many stimuli, especially stimuli that are used in cancer treatments. We feel that understanding the target genes that Egr1 regulates is an important goal and we will continue to work on the genes that have been revealed in our analyses. We can say at this stage that the target genes of Egr1 in breast cells differ according to the cell type, differ from prostate cells and differ from the target genes of Sp1 and p53 transcription factors (see Figure 1 in the Appendix). Once the validation of these results is completed, we will be able to publish this data.

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PERSONNEL WORKING ON DAMD17-01-1-0005

Eileen Adamson, PhD. PI, 15-20% 2001-2004
Ian de Belle, PhD. Post-doctoral Assoc.100% 2000-2001, 50% 2003-4
Thierry Virolle Post-doctoral Assoc., 15% 2002-2003
Jianxiu Yu, PhD, Post-doctoral Assoc. 100%, 1 yr Jan 2002-3
Hongyan Liang, PhD. Post-doctoral Assoc. 100% Jan 02-Aug 03

APPENDIX

Figure 1.
Cluster Analysis of the Egr1 bound to gene promoters under several conditions in MCF10A normal breast cells and MCF7 breast cancer cells. Comparisons with target gene that bound Sp1 or p53 transcription factors analyzed by chromatin immunoprecipitation with identification of putative target genes on the promoter array.

Figure 2.
Cluster Analysis of a portion of some of the genes in prostate cancer cells that were found bound to Egr1 transcription factor with the identification of distinct levels of each gene under different stimulatory conditions.:- NT = untreated; Serum-treated for 1 hour after starvation for 24 hours,; irradiated with 10 Gy ionizing radiation = IR; Treated with Etoposide for 2.5 hours = Eto; or irradiated with UV-C at 40 J/M^2 and collected 2 h later (UV). The red color indicates high binding levels and green low or no binding.

Transcription factors are shown in red, other cancer-related genes in blue.

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1. *Virolle, T., Adamson, E.D., Baron, V., Birle D., Mercola, D., Mustelin, T. and **de Belle, I.** (2001). The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling. Nature Cell Biology, 3: 1124-1128

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7. Moorehead RA, Hojilla CV, **de Belle I,** Wood GA, Fata JE, **Adamson ED**, Watson KL, Edwards DR, Khokha R. (2003), IGF-II regulates PTEN expression in the mammary gland, J. Biol. Chem. 278(50):50422-50427, 2003

Hierarchical clustering

Ward Method (average value) of ChIP data

Z scores

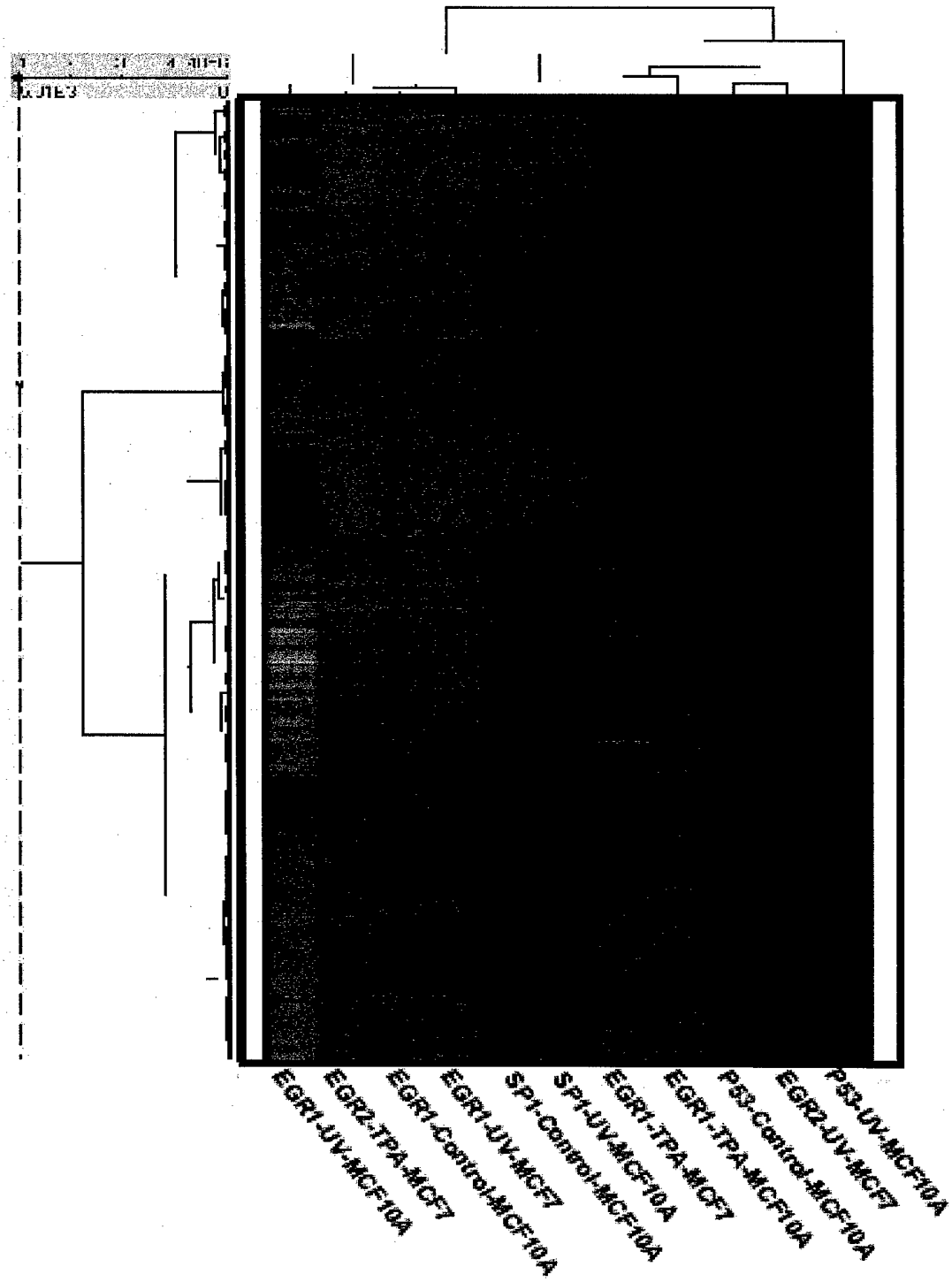
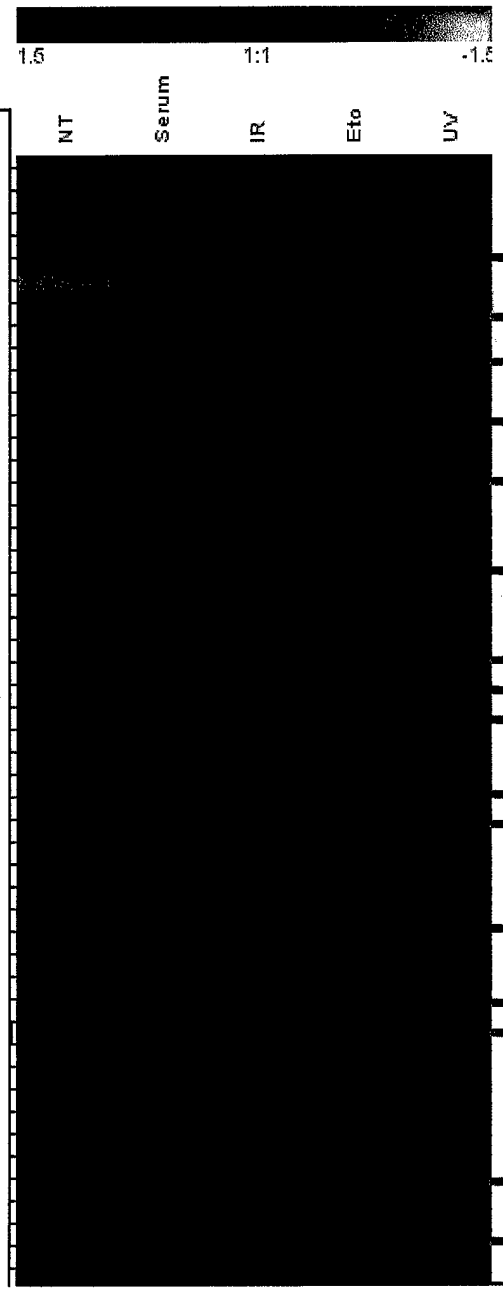


Fig. 3 Cluster Analysis

DU145 prostate cancer cells

1. Untreated
2. Serum
3 Ionizing radiation
4. Etoposide
5. UV-C radiation



Transcription factors
Cancer-associated

NM_003496	TRRAP
NM_004635	MAPKAPK3
NM_005036	PPARA
NM_005194	C/EBPbeta
NM_000551	VHL Promoter.
NM_001071	thymidylate synthetase
NM_002317	lysyl oxidase
NM_000323	RET protooncogene endocrine neoplasia
NM_003202	TCF7
NM_003242	TGFbetaIIR .
NM_002928	Gprotein a16
NM_004448	ErbB2
NM_002026	FN
NM_001238	Cyclin E1
NM_002229	JUNB
NM_001753	Caveolin 1
NM_001606	"ABCA2, ATP-binding transporter, Egr target"
NM_002160	Tenascin C .
NM_005229	"ELK1, member of Ets oncogene Fam"
NM_005252	Fos
NM_001909	Cathepsin D
NM_003029	SHC1
NM_002997	syndecan 1
NM_005940	Stromolysin 3
NM_002417	Ki67
NM_002151	"Hepsin , transmem protease"
NM_004832	GST Homolog .omega
NM_000926	Progesterone R
NM_005544	IRS1
NM_004472	FREAC-4 or FRKHL8
NM_005962	MAX interacting protein 1
NM_000039	Apolipoprotein A1
NM_000852	GST pi
NM_002982	MCP-1 monocyte chemoattraction prot
NM_007319	PSEN1
NM_001168	Survivin
NM_002658	"plasminogen activator, urokinase"
NM_000043	"tumor necrosis factor receptor superfamily, m
NM_014324	aMethylAcylCoA/Racemase

The Egr-1 transcription factor directly activates *PTEN* during irradiation-induced signalling

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The *PTEN* tumour suppressor[1] and pro-apoptotic[2] gene is frequently mutated in human cancers. We show that *PTEN* transcription is upregulated by Egr-1 after irradiation in wild-type, but not *egr-1*$^{-/-}$, mice *in vivo*. We found that Egr-1 specifically binds to the *PTEN* 5′ untranslated region, which contains a functional GCGGCGGCG Egr-1-binding site. Inducing Egr-1 by exposing cells to ultraviolet light upregulates expression of PTEN messenger RNA and protein, and leads to apoptosis. *egr-1*$^{-/-}$ cells, which cannot upregulate *PTEN* expression after irradiation, are resistant to ultraviolet-light-induced apoptosis. Therefore, Egr-1 can directly regulate *PTEN*, triggering the initial step in this apoptotic pathway. Loss of Egr-1 expression, which often occurs in human cancers, could deregulate the *PTEN* gene and contribute to the radiation resistance of some cancer cells.

The *PTEN/MMAC1/TEP1* tumour suppressor gene is mutated in a wide range of human cancers[3,4], and germline mutations of *PTEN* have been found in three inherited hamartoma tumour syndromes[5–7]. *PTEN* encodes a lipid phosphatase that removes the D3 phosphate from phosphatidylinositol-3-phosphate substrates, thereby inhibiting the generation of second messengers[8]. Deleting the *PTEN* gene in mice leads to embryonic lethality because of hyperproliferation of embryonic cells[9]. In Jurkat T leukaemia, breast and thyroid tumour cell lines, transient and inducible ectopic expression of PTEN induces cell-cycle arrest and cell death[10–12]. Expression of PTEN in PTEN-deficient multiple myeloma cells abolishes tumour growth *in vivo*[13]; PTEN phosphatase activity is essential for this effect[8]. Together, these findings suggest that loss of PTEN activity sensitizes cells to malignant transformation and imply that PTEN is a regulator of an important physiological pathway.

There have been no reports of transcriptional regulation of the *PTEN* gene to date, but a genomic fragment of DNA containing the full-length *PTEN* gene has been sequenced (K. Jensen *et al.*, unpublished results; GenBank accession number AF067844). We noticed that a 2-kilobase (kb) fragment upstream of the translation start site is highly GC-rich and contains several potential binding sites for the 'early growth response-1' (Egr-1) transcription factor; we tested these binding sites for activity. The hypothesis that Egr-1 (ref. 14) might regulate the transcription of the *PTEN* gene was attractive because the mutations in the two genes lead to some similar effects in cells, with variations depending on specific cell types. Like *PTEN*, the *Egr-1* gene also has growth-suppressing activities[15–19] and it was reasonable to think that *PTEN* could be regulated at least in part by Egr-1. A growing body of evidence indicates that Egr-1 is also required for apoptosis in some cells[20,21]. The results described here support the hypothesis that Egr-1 transactivates the *PTEN* gene to induce apoptosis.

Irradiation greatly upregulates production of Egr-1 (ref. 22). We used ultraviolet-C radiation to stimulate endogenous Egr-1 in 293T human fetal kidney and normal mouse mammary gland (NMuMG) epithelial cells to test whether *PTEN* expression was affected. Figure 1 shows that 4 h after irradiation with ultraviolet-C light at 40 J m^{-2} both PTEN and Egr-1 mRNAs (Fig. 1a, lanes 3 and 5) and protein (Fig. 1b, lane 2) were strongly upregulated, as was expression elicited by exogenous Egr-1 introduced by transient transfection (Fig. 1a, lane 2). This suggested that Egr-1 may act in the same signalling pathway as *PTEN*, and could be directly interacting with the *PTEN* promoter to effect this induction.

To find out how Egr-1 is able to regulate *PTEN* gene expression and what the mechanism might be, a 2-kb genomic DNA fragment corresponding to the *PTEN* promoter and its 5′ upstream regulatory sequences was amplified from human genomic DNA and cloned into a luciferase reporter plasmid thus creating *PTEN–luc*. This construct contained the full-length 5′ noncoding region of the mRNA. To determine whether the putative Egr-1-binding sites are involved in the regulation of the *PTEN* gene, *PTEN–luc* was transiently transfected into HEK293T, HT1080 fibrosarcoma (clone H4) or NMuMG cells with or without exogenous Egr-1 expression or with exposure to ultraviolet-C (40 J m^{-2}), γ-irradiation (5 Gy) or the DNA-damaging agent etoposide (20 μg ml^{-1}). Expression of *PTEN–luc* was well stimulated by exogenous Egr-1 expression, radiation or etoposide treatment in 293T (Fig. 1c) and NMuMG cells (Fig. 1d), but only ectopic Egr-1 was able to stimulate *PTEN–luc* expression in H4 cells, which lack Egr-1 (ref. 16; Fig. 1e). Using an antisense oligonucleotide to specifically inhibit Egr-1 expression blocked radiation- and etoposide-stimulated *PTEN* promoter activity (Fig. 1c, d). These results demonstrate not only that Egr-1 is able to stimulate *PTEN* promoter activity, but, more importantly, that Egr-1 is required for radiation- and etoposide-induced stimulation of *PTEN* expression. We also confirmed that the induction of *PTEN* promoter activity was dependent on the dose of Egr-1 transfected in 293T cells, which peaked at a fourfold induction (data not shown).

To determine whether *PTEN* transcriptional upregulation by Egr-1 occurs *in vivo*, wild-type and *egr-1*$^{-/-}$ mice were γ-irradiated and killed 2.5 h later, when Egr-1 induction is known to be high. Several tissues were rapidly dissected and flash frozen to analyse PTEN expression using reverse transcriptase–polymerase chain reaction (RT–PCR). Figure 1f shows that PTEN expression in wild-type mice was strongly increased in at least six tissues and moderately increased in three other tissues that have higher basal levels of

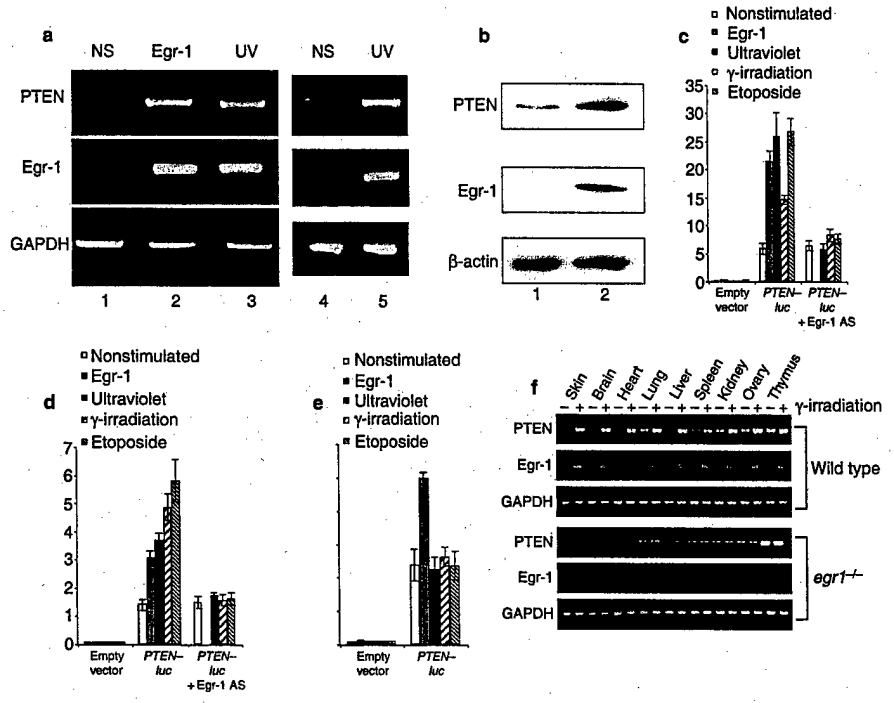

Figure 1 **Ultraviolet irradiation and ectopic Egr-1 stimulate expression of PTEN mRNA and protein. a**, Analysis of PTEN and Egr-1 mRNA expression levels by semi-quantitative RT-PCR assay with total RNA extracted from 293T nonstimulated (NS) cells as a template (lane 1); from 293T cells expressing exogenous Egr-1 (lane 2); ultraviolet-stimulated 293T cells (lane 3); nonstimulated mouse mammary cells.(lane 4); and ultraviolet-stimulated mouse mammary cells (lane 5). Amplification of GAPDH mRNA was monitored as an internal control. **b**, Immunoblot analysis for PTEN was performed with protein extract from nonstimulated (lane 1) and ultraviolet-stimulated NMuMG cells (lane 2), using a specific PTEN antibody as described in the Methods. Expression of β-actin was monitored as an internal control. **c–e**, The full-length *PTEN* 5′ promoter–reporter construct (*PTEN–luc*) was transiently transfected with or without 0.2 μM antisense (AS) Egr-1 oligonucleotides into 293T cells (**c**), NMuMG cells (**d**) or HT1080 cells (**e**), which do not express Egr-1, stimulated by ultraviolet irradiation, γ-irradiation, etoposide or exogenous Egr-1. Luciferase activity was determined as described in the Methods. Empty vector luciferase values correspond to the background expression of the pGL3 basic empty reporter gene. The luciferase activity values and error bars reflect the average and the standard deviation between at least three separate experiments with six replicates. **f**, Analysis of PTEN and Egr-1 mRNA expression levels by semi-quantitative RT-PCR, in nine different tissues from wild-type and *egr-1*$^{-/-}$ mice that were or were not γ-irradiated with 5 Gy and killed 2.5 h later.

PTEN mRNA. Corresponding with the radiation-induced PTEN, the expression of Egr-1 was induced about tenfold in most tissues (Fig. 1f, row 2). In contrast, Egr-1 expression in tissues from Egr-1-deficient mice was undetectable and uninducible (Fig. 1f, row 5) and PTEN expression was detectable but was uninduced by radiation (Fig. 1f, row 4). The analyses were performed with equal amounts of mRNA, as shown in the levels of the control mRNA from the GAPDH gene. These results indicate that Egr-1 is a major inducer of the *PTEN* gene *in vivo*.

The full-length *PTEN* promoter has numerous putative Egr-1-binding sites. To determine which sites might be responsible for Egr-1 stimulation, a series of deletions were made. The translation start site was designated as +1 for this study. The first three 5′ or distally truncated *PTEN* promoter constructs had similar stimulatory activity compared with the full-length promoter (data not shown), as did fragment Δ5′, which contained the proximal half of the sequences tested (−1 to −1031; Fig. 2a, b). Both transiently transfected Egr-1 and endogenous Egr-1 stimulated by ultraviolet irradiation of the cells produced very similar transactivating activity. Furthermore, the deletion of most of the 3′ end of these cloned regulatory sequences from the position −1 to −779 (the Δ3′ construct) did not abolish this stimulation. This narrowed the putative active binding sites to the remaining sequences located between nucleotides −779 and −1031, which includes the start of transcription. This fragment (*min PTEN–luc*) retains full activity (Fig. 2a, b). This fragment contained a 117-base pair (bp) GC-rich region containing three putative Egr-1-binding sites. Deleting this region in the context of the full-length *PTEN* regulatory sequences eliminated stimulation, narrowing the search to this short piece of DNA (Fig. 2a, b).

The three Egr-1-binding sites (EBSA, EBSB and EBSC) shown in Fig. 2c were individually mutated to give the mut A, mut B and mut C constructs. Mutation of EBSB and EBSC did not affect induction of the *PTEN* promoter in response to ultraviolet light or exogenous Egr-1 expression, whereas mutation of EBSA abolished the effect of both (Fig. 2d). These results demonstrate that the nine nucleotides GCGGCGGCG located between positions −947 and −939 constitute a functional *cis*-acting element necessary and sufficient for *PTEN* promoter stimulation by both transiently transfected Egr-1 and endogenous Egr-1 stimulated by ultraviolet irradiation.

The specificity of Egr-1 binding to the *PTEN* promoter was confirmed by *in vitro* gel-mobility shift assays using a 27-bp probe containing the normal or mutated EBSA site. Supershift assays with antibodies to Egr-1 confirmed the identity of Egr-1 binding to the probe (data not shown).

In order to assess whether direct binding of Egr-1 to the 117-bp portion of the endogenous *PTEN* regulatory sequences occurs in intact cells[23], we performed chromatin crosslinking studies on liv-

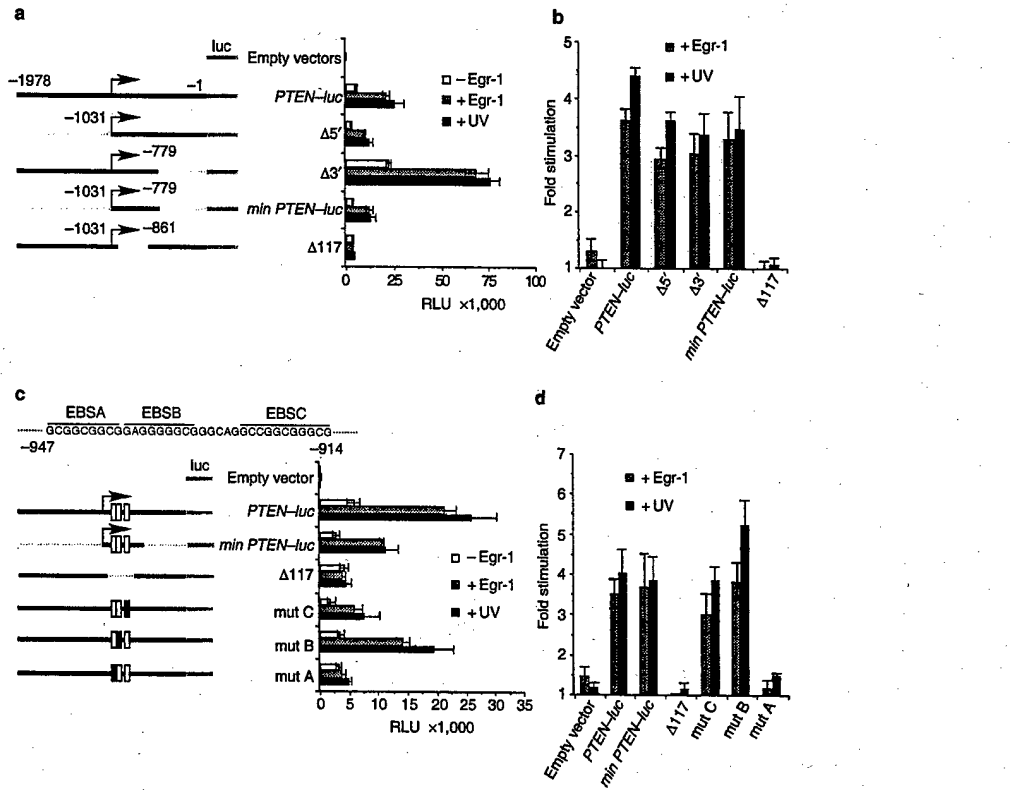

Figure 2 **Mapping the Egr-1-responsive element in the *PTEN* promoter.** **a**, Full-length and deleted *PTEN* regulatory sequences are represented on the left. The numbers indicate the position of the deleted fragments relative to the translation start site (+1). An arrow represents the start of transcription. Right, *PTEN–luc* and the deleted constructs were transiently transfected into 293T cells stimulated or not with ultraviolet irradiation or ectopic Egr-1 expression. Luciferase activities were assayed as described in the Methods and are shown as absolute values of relative luciferase units (RLU). **b**, Ultraviolet and Egr-1 stimulated activity. The values (x-fold stimulation) are derived from the ratio between the basal and the Egr-1-induced promoter activities shown in **a**. **c**, A GC-rich region in the *PTEN* 117-bp fragment corresponding to the three putative Egr-1-binding sites (EBSA, EBSB and EBSC). Left, unmutated EBSA, EBSB and EBSC are represented in the context of the full-length promoter by open boxes and mutated versions by black boxes. Right, the wild-type and mutated constructs were transfected into 293T cells stimulated or not by ultraviolet or exogenous Egr-1, and assayed as described in the Methods. **d**, Ultraviolet- and Egr-1-stimulated activity represented as the ratio (x-fold stimulation) between the basal and the Egr-1-induced absolute values of the promoter activities assessed in Fig. 2c.

ing cells, recovering Egr-1-binding sites by immunoprecipitation (see Methods). Three different conditions of 293T cells were tested: cells transfected with empty expression vector without ultraviolet irradiation was a control; a similar sample irradiated to induce endogenous Egr-1; and cells transfected with an Egr-1 expression vector. Egr-1 became fixed to its DNA target sequences after chromatin crosslinking, allowing recovery of the Egr-1-bound genomic DNA fragments by specific Egr-1 immunoprecipitation. We used a nonimmune serum as a negative control. The detection of the *PTEN* genomic fragment among all the captured fragments was made by PCR amplification using a pair of specific primers located at each end of the 117-bp sequence containing the Egr-1-binding site. Cells transfected with Egr-1 or ultraviolet-irradiated yielded an amplified product that showed the same migration pattern (Fig. 3, lanes 5, 6) as the 117-bp *PTEN–luc* control fragment (Fig. 3, lane 7). In contrast, no amplification was found either for the nonirradiated cells containing empty vector (Fig. 3, lane 4) or for the control nonimmune serum immunoprecipitated samples (Fig. 3, lanes 1–3). Each PCR band was purified and its identity verified by DNA sequence analysis. The sequences of the amplified PCR bands were identical to the 117-bp region in the *PTEN* promoter (data not

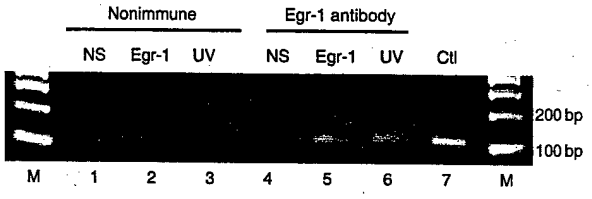

Figure 3 **Egr-1 binds directly to *PTEN* regulatory sequences in vivo.** Nonstimulated 293T cells (NS, lanes 1 and 4), 293T cells stimulated by Egr-1 (lanes 2 and 5) or ultraviolet light (lanes 3 and 6) were chromatin crosslinked and then immunoprecipitated[23] with a specific Egr-1 antibody or a nonimmune control antibody. The detection of the *PTEN* GC-rich 117-bp captured fragment was performed by PCR as described in Methods. Lane 7 corresponds to the control (Ctl) 117-bp *PTEN* fragment directly amplified from *PTEN–luc*. Lanes 1, 2 and 3 show the PCR amplification from the control nonimmune immunoprecipitation and lanes 4, 5 and 6 correspond to the *PTEN* 117-bp captured fragment amplified from the Egr-1-specific immunoprecipitation. M, marker.

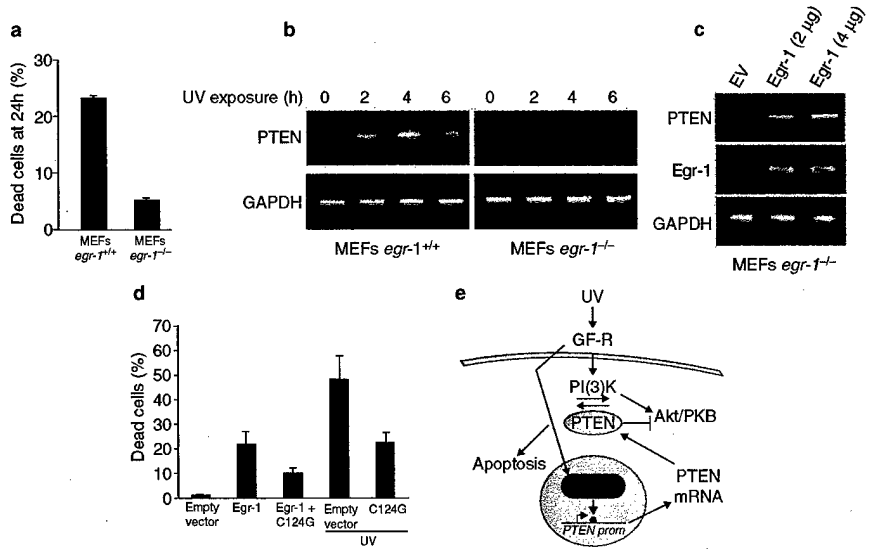

Figure 4 **Egr-1 is required for PTEN-dependent ultraviolet-induced apoptosis. a**, Monolayers of wild-type and Egr-1-null mouse embryo fibroblasts (MEFs) were exposed to ultraviolet-C radiation as described, and 24 h later the ratios of fragmented nuclei were compared (means of two experiments). **b**, MEFs collected at the indicated times after irradiation were assayed for PTEN mRNA levels by semi-quantitative RT-PCR. **c**, Dishes (60 mm) containing *egr-1*$^{-/-}$ MEFs were transfected with 2 or 4 μg Egr-1 expression vector or empty vector (EV) and 30 μl of lipofectamine. The day after transfection the cells were collected and PTEN mRNA levels were assayed by semi-quantitative RT-PCR. **d**, Inhibition of PTEN activity decreases the sensitivity of the cells to ultraviolet-induced cell death. 293T cells were transfected with the expression vectors shown in the absence of irradiation, or irradiated with or without overexpression of the catalytically inactive form of the PTEN protein (Cys 124→Gly). Empty vector was transfected as a negative control. Dead cells were determined 24 h later by trypan blue staining. Detached and trypsinized cells were pooled and incubated with 0.2% trypan blue. Cell death is shown as percentage of blue cells. **e**, The signalling events leading to apoptosis in 293T and NMuMG cells. The red circle represents the Egr-1-binding site; GF-R, growth factor receptor; PI3K, phosphoinositide 3-kinase; Akt/PKB, Protein kinase B.

shown), demonstrating that this sequence is an Egr-1 target in living cells. This result is consistent with the *in vitro* data and clearly demonstrates that Egr-1 binds directly to the genomic *PTEN* 5′ noncoding region in cells that have been ultraviolet irradiated, as well as in cells that overexpress exogenous Egr-1.

In 293T cells, the *PTEN* gene is intact, because exogenous Egr-1 or ultraviolet-C irradiation induce PTEN mRNA and protein, followed by apoptosis 24 h later. It has been shown that ultraviolet irradiation leads to apoptosis of 70% of *pten*$^{+/-}$ mouse embryo fibroblasts (MEFs), but *pten*$^{-/-}$ MEFs survived[24] indicating that the loss of PTEN activity probably causes resistance to ultraviolet-induced apoptosis. Therefore, we tested *egr-1*$^{-/-}$ MEFs in which ultraviolet irradiation does not induce PTEN mRNA (Fig. 4b) and found that these cells were also resistant to apoptosis induced by ultraviolet-C irradiation (Fig. 4a). The introduction of exogenous Egr-1 into the *egr-1*$^{-/-}$ MEFs restored the stimulation of *pten* gene expression (Fig. 4c). In wild-type MEFs, high levels of Egr-1 and PTEN mRNA expression were induced 2–4 h after irradiation (Fig. 4b), and a significant proportion of cells died within 24 h. Furthermore, inhibiting endogenous PTEN activity by overexpressing the catalytically inactive form of PTEN[10] (the Cys 124→Gly construct) leads to a 50% decrease of the sensitivity of 293T cells to ultraviolet-induced apoptosis mediated by Egr-1 (Fig. 4d). Because both the absence of Egr-1 and the inhibition of PTEN activity allow the cells to survive this death signal, we conclude that Egr-1 induction of *pten* is required for ultraviolet-induced apoptosis.

Therefore, the Egr-1 transcription factor directly transactivates the *PTEN* gene and is responsible, at least in part, for the apoptotic response after *PTEN* is induced by radiation or etoposide. Loss of Egr-1 may therefore contribute to radiation resistance of some cancer cells. □

Methods

Transfection, ultraviolet/γ-irradiation and etoposide treatment

Cells were seeded into 48-well plates at the density of 12,000 cells per well one day before transfection. The transfection was performed with the Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN). Typically 250 ng of total plasmid DNA (100 ng of reporter construct, 100 ng of Egr-1-expressing vector, and 50 ng of empty pcDNA3 vector) were mixed with 1 μl, 2 μl and 3 μl of Fugene 6 respectively for 293T, H4 and NMuMG cells. Cells were collected 24 h after transfection and luciferase activities were assessed using the Luciferase Assay System (Promega, Madison, WI) and an EG&G Berthold LB96P luminometer (PE Biosystems, Wellesley, MA). Cells were ultraviolet-C irradiated (40 J m^{-2}) in a Stratalinker (Stratagene, La Jolla, CA), γ-irradiated (5 Gy) or treated with 20 μg ml^{-1} etoposide to induce a response that includes high and transient induction of Egr-1 (ref. 25). After treatment the cells were incubated 4 h before harvesting.

To block Egr-1 expression we used a 20-mer-phosphorothioate analogue antisense oligonucleotide highly specific to Egr-1 (sequence available upon request).

MEFs derived from *egr-1*-null mice[26]

Dermal fibroblasts from skin explants from *egr-1*$^{-/-}$ and *egr-1*$^{+/-}$ mouse embryos were cultured as described previously[27] to derive 3T3-type cells. MEFs (56 population doublings) were seeded at 1 × 10^6 per 60-mm dish overnight, irradiated with ultraviolet-C light at 40 J m^{-2} and harvested at 0, 2, 4 and 6 h for RT-PCR analysis of Egr-1 and PTEN (see below). Duplicate dishes containing 5 × 10^6 cells were cultured for 24 h after irradiation and apoptosis was estimated by staining with propidium iodide and counting the proportion of cells with fragmented nuclei.

Plasmid construction

We amplified 5′ *PTEN* regulatory sequences by PCR from genomic DNA using a pair of appropriate primers (5′-*Kpn*I-GCCGGGTTTCACGCGGC-3′ and 5′-*Hind*III-GTCTGGGAGCCTGTGG-3′) located respectively at the position −1 and −1,978 from the ATG. The amplified product was purified and cloned into the *Kpn*I/*Hind*III-digested pGL3 basic reporter gene to give the *PTEN–luc* construct. The Δ5′ construct was amplified by PCR from *PTEN–luc* using the primers 5′-*Kpn*I-CCTCCCCTCGCCCGGCGCGG-3′ and 5′-*Hind*III-GTCTGGGAGCCTGTGG-3′. The amplified product was purified and cloned into the *Kpn*I/*Hind*III-digested pGL3 basic reporter gene.

The Δ3′, min *PTEN–luc*, Δ117, mut A, mut B and mut C constructs were made by directed mutagenesis according to the Quick-Change kit protocol (Stratagene). To create Δ3′ and Δ117, respectively 778 bp and 117 bp were deleted from *PTEN–luc* using the oligonucleotides 5′-GAGTTGAGCCGCTGTGAGGCGAGGCAAGCTTGGCATTCCGGTACTGTTGG-3′ and 5′-CTCGGTCTTCCGAGGCGCCCGGGCGCGGCAGGATACGCGCTCGGCGCTG-3′, respectively. To create the min *PTEN–luc* construct, 778 bp were deleted from Δ5′ using the oligonucleotide 5′-GAGTTGAGCCGCTGTGAGGCGAGGCAAGCTTGGCATTCCGGTACTGTTGG-3′. To create mut A, mut B and mut C constructs, the *PTEN–luc* EBSA, EBSB and EBSC were changed into *Eco*RI restriction sites using respectively the

following oligonucleotides 5′-AGGCGCCCGGGCTCCCGGCGAATTCGCG-GAGGGGGCGGGCAGGCCGGCGGGCGGTGATGT-3′, 5′-AGGCGCCCGGGCTCCCGGCGCG-GCGGCGAATTCATTCGGCAGGCCGGCGGGCGGTGATGT-3′ and 5′-GGCGCGGCGGCG-GAGGGGGCGGGCAGAATTCGAATTCGTGATGTGGCAGGACTCTTTATG-3′. All the DNA plasmid constructs were purified using columns from Qiagen (Valencia, CA). The C124G catalytically inactive form of PTEN was made as described elsewhere[10].

RT-PCR and western blots

Both total RNA and proteins were purified from 293T cells using the TRIzol reagent (Life Technologies) according to the manufacturer's protocol. When the cells were ultraviolet irradiated, the extractions were done 4 h after treatment. RT-PCR was performed with 200 ng of total RNA as template using the superscript RT-PCR kit reagent according to manufacturer's instructions (Life Technologies, Rockville, MD). PTEN, Egr-1 and control GAPDH mRNAs were reverse transcribed and amplified using the specific primers: 5′-GACAGCCATCATCAAAGAGA-3′ and 5′-TGACGGCTCCTC-TACTGTT-3′; 5′-CCCAGCTCATCAAA-3′ and 5′-CACCACACTTTTGTC-3′; 5′-AACCATGAGAAG-TATGACAAC-3′ and 5′-GTCATACCAGGAAATGAGCT-3′, respectively. The amplified products were resolved on a 2% agarose gel.

For the western blot analyses, after extraction, the proteins were boiled for 3 min and separated with 10% SDS–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to Immobilon P membranes (Millipore Corporation, Bedford, MA), blocked, reacted with primary antibodies at 1 μg ml^{-1} and an anti-rabbit secondary antibody conjugated to horseradish peroxidase for enhanced chemiluminescence detection of the signals (Amersham, Grand Island, NY).

Crosslinking and identification of Egr-1 bound to DNA

Live cells were treated with formaldehyde to crosslink chromatin complexes and Egr-1-containing fragments were recovered by immunoprecipitation as described elsewhere[23]. Identification of the captured *PTEN* 5′ regulatory sequences was performed by PCR analysis using the primers 5′-CTCGGTCTTCC-GAGGC-3′ and 5′-CCGAGCGCGTATCCTG-3′. Two consecutive rounds of 30 cycles each of PCR were performed, using the captured fragments as templates. The bands were excised from the gel used to analyse the product, purified and sequenced using an ABI 377 sequencer system (ABI, Foster City, CA).

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Review

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Egr1 Transcription Factor: Multiple Roles in Prostate Tumor Cell Growth and Survival

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Key Words
Prostate cancer · Egr1 binding · Egr1 regulated · Gene promoters · Growth factors · Angiogenesis

Abstract
The transcription factor, Egr1, so-called because it is encoded by the immediate early growth response gene, *Egr1*, is rapidly induced by growth factors to transduce the proliferative signal. The induction of Egr1 by external stimuli is generally transient but appears to be sustained in some prostate tumor cell lines and tumors, suggesting that Egr1 stimulates tumor cell growth. In contradiction, in breast, lung and brain tumors, Egr1 expression is often absent or reduced and when re-expressed, results in growth suppression. Re-expression of Egr1 in tumor cells also leads to antiapoptotic activity, which would encourage tumor cell survival. Egr1 is also required for, or stimulates, the differentiation of several cell types. Another contradiction is that after stress stimuli to some cell types, Egr1 is required for programmed cell death or apoptosis in both normal and tumor cells. Egr1 also plays a role in tumor progression, through the hypoxic signal generated in growing tumors. Egr1 is highly induced under these conditions and its activities stimulate angiogenesis and improved survival of tumor cells. How this large agenda can be achieved lies in the choice of Egr1 target genes, and varying patterns of coordinated expression have been described, but the mechanisms for this choice are not clear. This review points to areas where research should be focussed.

Introduction

The Egr1 *gene: Egr1* is also known as *NGFI-A, Zif268, Krox 24* and *Tis8* [1–5] and is located on chromosome 5q31 in a region that is often deleted in acute myeloid leukemia patients. Gene losses that lead to tumors are usually attributed to tumor suppressors, and indeed, characteristics of a tumor suppressor gene have been recorded for Egr1. The loss of Egr1 expression occurs in several types of tumor cell lines such as breast, lung and glioma/astrocytoma [6–8].

There are three other original members of the family, Egr2, -3, and -4. All are inducible by growth factor stimuli and all are transcription factors that can bind to the same nucleotide sequence. A related gene, *Egr-α/BTEB* was described more recently [9, 10] and all family members have considerable homology in the zinc finger DNA-binding region but with much less homology in other regions. This family may be regulated by Egr1 [11] and binding sites are present in the promoters of BTEB1 and 2. The tumor suppressor gene, Wilms' tumor gene, *WT1*, is related also by virtue of its DNA binding site preference for

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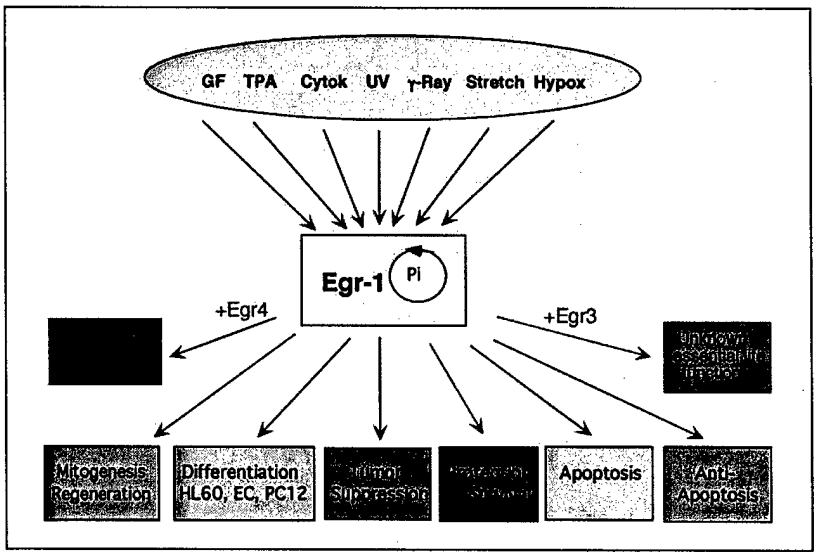

Fig. 1. Some of the numerous stimuli that elicit the expression of the Egr1 gene are shown. The resulting effects on cells depend mainly on the cell type, as well as the strength and duration of the stimulus and the co-stimulation of feed-back mechanisms.

the same or similar GC-rich 9 bp sites with the consensus sequence GCGG/TGGGCG. Two genes, NGFI-A binding proteins 1 and 2 (NAB1 and 2), inhibit the activities of the Egr family and NAB2 acts as a negative feedback inhibitor of growth-factor-stimulated Egr1 and Egr3 [12–15]. There is also evidence that the Egr family members autoregulate each other [16]. To learn more of the general characteristics of the *Egr* family of genes, consult published reviews [17–24]. The activities of the extended family members may account for some of the variations in responses made by cells.

The Egr1 Protein

Egr1 is a (calculated) 59-kD polypeptide that migrates with an M_r of 80 kD during electrophoresis. It becomes located in the nucleus through its carboxy-terminal zinc finger domain and a basic nuclear localization domain upstream of the zinc fingers. Egr1 has many phosphorylation sites and is normally short-lived except when phosphorylated. Several domains in Egr1 have been recognized, in addition to the zinc finger DNA-binding domain: there are two strong activation domains, two less active regions and a repressive domain in the region close to the DNA binding region. Egr1 binds to DNA as a monomer and does not appear to *require* the presence of any other known protein to have a positive effect on transcription. The transcriptional activity of Egr1 is modified by binding to two negative regulatory proteins, NAB1 and NAB2, at the repressive domain [12, 25]. The *NAB2* gene is also induced by growth factors and stress stimuli and clearly affects the net responses of cells to these stimuli including the inactivation of Egr1 after the initial induction [26]. Figure 1 indicates some of the cellular responses to Egr1 after its induction by a variety of stimuli.

The Egr1 family of proteins bind to target sites which are often close to the start of translation and often within the 5′ untranslated region in gene promoters. The similarities of binding sites indicate that each member of the family may interact with the same binding site in gene promoters, but have different affinities and hence different effects. For example, Egr1 and WT1 can produce opposite effects when they bind to the same promoter elements in target genes [27]. Egr1 overexpression causes enhanced growth and WT1 expression exerts an antagonizing effect in baby rat kidney cells [28]. Egr1 when induced, often displaces Sp1 or Sp3, other 3-zinc-finger gene transcription factors, from common or overlapping GC-rich binding sites, where Sp1 and Sp3 support basal expression of genes [29, 30]. Several factors bind physically to Egr1 and have been postulated to affect its transactivating roles: p53, Sp1, NF-κB and p300/CEBP have all been found to bind. These are nicely discussed in the context of the regulation of endothelial cell gene activities in a recent review [31]. Together with the interactions of NAB1 and NAB2 with Egr1, these proteins form the basis for our current understanding of the complex mechanisms that guide transcriptional modulation of target genes by Egr1.

The Egr1 *Knockout Mouse*

Unexpectedly, the abrogation of the *Egr1* gene in vivo was not lethal, and two knockout mouse models [32–34] show somewhat differing effects on the fertility of adult mice. Due to redundancy between the different members of the family, sufficient Egr1-like activity is retained when only one member of the family is inactivated. Crosses between Egr1 and Egr4 knockout mice indicated the importance of the luteinizing hormone-β subunit (LH) as a target gene in the pituitary, with redundancy between Egr4 and *Egr1* in regulating LH production in male mice [35]. While the Egr3 knockout and the *Egr1* knockout produce live adult mice, the combination of Egr1 and Egr3 losses produces mice that die at birth [Jeffrey Milbrandt, Washington University, St. Louis, Mo., USA, pers. commun.], indicating another important pair of shared but as yet unspecified activities. In spite of the overlapping functions of the members of the family, knockout and transgenic animal models have proved useful in determining the essential role of Egr1 in protective stress responses in the lung vasculature, in the heart and in the kidney [36–40].

Egr1 Is Associated with Growth Promotion and/or Survival

Mouse Models Demonstrating the Regulation of Tumor Growth by Egr1

Egr1 is transcriptionally upregulated upon the addition of many growth factors and oncogenes to cells, leading to the categorization of Egr1 as a promitotic gene. It has been established using mouse models, that in the prostate the progression of tumors is upregulated, at least in part, by Egr1. The prostate tumor mouse model (TRAMP mice) established by Greenberg et al. [41], wherein the SV40 large T tumor antigen is expressed from the rat probasin promoter allows mechanistic studies on the genesis of prostate cancer. In these transgenic mice, palpable tumors are formed between 10 and 38 weeks of age, accompanied by elevated fibroblast growth factor (FGF) and insulin-like growth factor (IGF) levels. These growth factor activities are increasingly detected as the tumor progresses and metastases occur [42, 43]. Since levels of Egr1 are high in prostate cancer [44–46] while the repressor protein NAB2 levels are low, the effects of lack of Egr1 in these mice and another prostate cancer model of transgenic mice [47] were recently tested. Deficiency of Egr1 did not affect the initiation and growth rates of prostate cancer, but the progression of the disease was strongly affected, significantly delaying the incidence of invasive carcinoma in both animal models. The results indicate that deficient Egr1 in either prostate epithelial or in neuroendocrine cells of the prostate gland significantly reduces cancer progression [48]. The expression of platelet-derived growth factor-A (PDGF-A) and transforming growth factor β1 (TGFβ1) (both known target genes of Egr1 and known stimulators of prostate cancer) are expressed at 30- to 60-fold higher levels in late tumors compared to the earlier tumors in the $Egr1^{-/-}$ mice, suggesting that these growth factors are driving the progress of the disease [48].

Egr1 Takes Part in Mitogenic Signaling in Prostate Cancer in the Human

Egr1 is correlated with increased growth and malignancy in prostate cancer. Egr1 mRNA was quantified in 96 prostate specimens representing different Gleason scores and 10 benign tissues showing no histological manifestation of benign prostatic hypertrophy using in situ hybridization. Egr1 mRNA was expressed at significantly higher levels in cancer than in normal prostate ($p < 0.001$). In cancer with Gleason scores 8–10, the expression of Egr1 was higher compared with those of lower Gleason scores ($p < 0.005$). Immunohistochemical staining showed predominately basal cell nuclear Egr1 protein in prostatic acini. Nuclear staining was weak in nonmalignant tissues, more intense in moderately differentiated carcinoma, and most intense in poorly differentiated carcinoma [46]. Egr1 mRNA levels were found to be elevated in 12 of 12 intraprostatic adenocarcinomas but not in breast or ovarian cancers, or in rapidly dividing rat ventral prostate cells [45]. The connection between Egr1 activity in prostate tumor is substantiated by several studies that point to genes that are regulated by Egr1 and that are direct targets for transactivation (table 1). However, most genes that express with good correlation to Egr1 expression may not be direct targets of Egr1 and this evidence is beginning to be collected.

Egr1 as a Tumorigenic Factor via IGF-II and IGF-IR Regulation in Prostate Cells

Several growth factors and their receptors are thought to play roles in prostate cancer, especially insulin-like growth factors IGF-I and -II and their receptors that have been identified as important growth-promoting genes in the prostate gland. Whether Egr1 plays a role in prostate cancer development through deregulation of the 'IGF axis' leading to cell proliferation, motility and tumor progression has been partially examined. For several tumor cell types including prostate, hepatoma, pancreatoma and

Table 1. EGR1 known and putative target genes

Gene	Prom. access.	Method of analysis	Tissue	Comments	Reference
Proliferative/transforming					
Amphiregulin	M30698	L1/WT1 only	kidney	WT1 correl. with high Ar exp. on arrays	57
Cyclin D1	Z29078	L2/Egr1	squamous epithelial cells	Egr1 upregulates	71
EGF-receptor (HER1)	X06370	L2/WT1 only	Wilms' tumor/PC12 cells	WT1 represses	55, 88
Elk-1	Y11432	L2/Egr1	premonocytic cell lines	Egr1 induces	89
FGF2, basic FGF	Y13468	L2/Egr1	rat astrocytes	Egr1 induces	66
G protein a-i2	X07854	L2/Egr1 and WT1	kidney cells	Egr1 induces, WT1 represses	90, 91
ID-1transcription factor	U57645	L2/Egr1	myoblasts and fibroblasts	Egr1 induces	92
IGF-I	M12659	L1 for WT1	prostatic stromal cells	WT1 may repress	93, 94
IGF-II	X03562	L2/Egr1	hepatogenesis	Egr1 induces	49, 50, 95
IGF-I receptor	M69229	L1/WT1, high in tumor	Wilms' tumor	WT1 represses	52, 96
IGF-II receptor	X91875	L1/WT1	prostate cells	WT1 represses	97
IGF-binding protein 2	S37712	L1/Egr1	two tumor lines	basic exp. high	98, 99
c-Jun/(JunB/JunD?)	J04111	suggested by GC-rich	HeLa cells	UV induced	100
Ornithine decarboxylase	S71124	L2/WT1 only	hepG2	WT1 represses	101
Thymidine kinase	X15509	L2/Egr1	transient transfection	Egr1 induces	72
PDGF-A	M59423	L2/Egr1	transient transfection	Egr1 induces, WT1 represses	102, 103
PDGF-B	X02811	L2/Egr1	injury to rat aorta	Egr1 displaces Sp1	104
PRL-1	AF051160	L2/Egr1	liver regeneration	Egr1 induces	105
Survival/differentiation					
ApolipoA	J00098	L2/Egr1	HepG2: liver regeneration	Egr1 induces, leading to inc. HDL-Chol	40, 106
Bag1	NM004323	GC-rich	antiapoptotic, increases cell motility of gastric cancer cells		107, 108
BCl2	XM_036929	L2/Egr1/WT1	HT1080 fibrosarc. cells	Egr1 supp. and WT1 induces BCl2	109, 110
E-cadherin	D49685	L2/WT1	NIH3T3 cells	WT1 induces	111
CD44	M69215	L2/Egr1	B lymphocytes	Egr1 induces this homing and mig. protein	112
Coll 1α2	AB004317	L2/Egr1	osteoblast cells	Egr1 represses	113
Fibronectin	M15801	L2/Egr1	glioblastoma cells	Egr1 induces. Progression of prostate CA	78, 79
I-CAM	X59286	L2/Egr1	B lymphoma cells	Egr1 induces	114
Laminin β2	Z68155	L1/Egr1	cardiac fibroblasts	Egr1 correlated with laminin	115
Luteinizing hormone-β	not found	Egr1 KO fem. infertile	pituitary	Egr1 induces LH	33
NF-κB	L01459	L2/Egr1	T cells, fib. and breast arrays	Egr1 induces RelA and survival	116, de Belle, unpub.
PGP2/MDR1b	L07624	L2/Egr1/Sp1 competition	rat hepatoma cells	Sp1 induces, Egr1 represses the MDR1b	117
Apoptosis					
p53	X54156	L2/Egr1	tumor cells	Egr1 induced p53 leading to apoptosis	118, 119
Clusterin (TRPM-2)	M63376	High in prostate CA	early marker and apoptosis	stress and TGFβ-induced	120, 121
PARP	NM_001618	GC-boxes	poly(ADP-ribose) poly'ase	DNA repair. Substrate for apoptosis	122
PTEN	AF067844	L3/Egr1	several cell lines	Egr1 induced PTEN	141
TNF-α	U42625	L3/Egr1	monocyte cell line	Egr1 induces TNFα	123, 124
Tumor progression/angiogenesis					
MT1-MP-metallo-protease	not found	L2/Egr1	endothelial cells	Egr1 induces MT1-MP	125
FLT1	D64016	L2/Egr1	macrophage/endothelial cells	Egr1 induces FLT-1	126, 127
Gelatinase MMP9	XM_012503	L1/TNFα induced	skin, bone tumor	TNFα induces MMP9 via Egr1?	128, 129
Hepsin	X07732	high in prostate CA	potential tumor marker	unknown	80, 81, 82, 130
IGF-II	X03562	L2/Egr1	hypoxia in hepG2 cells	Egrl induced P3 of IGF-II	49
TF	NM_001993	L3/Egr1	monocyte cell line	Egr1 induces/NAB2 inhibits angiogenesis	26, 131
uPA	Y11873	L1/GC-rich	PMA stimd. HepG2, lung CA	TNFa induces uPA via Egr1?	83, 132
uPAR	S78532	L2/Sp1	endothelial cells	PMA stimd. uPAR exp.	133, 134
VEGF	AF095785	L2/Egr1	vasc. endo. cells and hypoxia	Egr1 induces coord. with HIF-1α	135, 136

Table 1 (continued)

Gene	Prom. access.	Method of analysis	Tissue	Comments	Reference
Growth inhibitors					
p21/WAF1	U24170	L1/Egr1	tumor cells	Egr1 correlates with inc. p21	137
p57/KIP2	D64137	L1/Egr1	array data/prostate cancer/ Wilms' tumor		44, 138
PTP1B	AY029236	L2/Egr1	suppresses transformation	Egr1 represses PTP1B	30
TOE1	not listed	L3/Egr1	suppresses growth	Egr1 induces TOE1	de Belle, unpub.
Thrombospondin	J04447	L2/Egr1	mouse NIH3T3 cells	Egr1 stimulates TSP growth inhibitor	139
TGFβ	J04431	L3/Egr1	tumor/endothelial cells	Egr1 induces TGFβ	140

The listed genes have been grouped to provide a possible phenotype indicating possible roles in tumorigenesis. The level of certainty that each is an Egr1 target gene is indicated by the method of analysis used. Level 1 (L1) means that there is a correlation of Egr1 or WT1 expression with the indicated gene. This includes endogenous putative target genes that have been shown to be induced by the overexpression of Egr1. Level 2 (L2) indicates that the promoter of the tested gene was shown to bind Egr1/WT1 and /or to affect the activity of a promoter-reporter vector. Level 3 (L3) means that the binding of Egr1/WT1 to its binding site(s) was verified by recovery of the promoter fragment by PCR, after cross-linking with formaldehyde, as described [87].

breast cancer, IGF-II is a potent growth factor capable of inducing tumor progression and it can be upregulated by Egr1 [49]. In fetal development of the kidney, IGF-II must be downregulated by WT1 to obtain epithelial differentiation [50], a process that is aberrantly regulated in Wilms' tumor, a pediatric kidney cancer caused by genetic inactivation of the *WT1* gene [51]. During development, IGF-I is expressed the highest at the early blastema growth stage when Egr1 is present and WT1 is absent. WT1 is over-expressed in prostate cancer and appears to be responsible for the repression of the *IGF-IR* gene [52] which would be antiproliferative. But since Egr1 is also overexpressed and may induce the *IGF-IR* gene, there might be dominance of each gene at different times, although this remains to be determined. The activity of the IGFs is also modified by a number of binding proteins that inhibit their activities. IGFBP-3 is thought to be important as a carrier and protector of the IGFs, suggesting a complex system of regulation. All of these genes carry putative Egr1-binding sites in their promoters and this question can be readily tested.

Does Egr1 Regulate the Epidermal Growth Factor Receptor-Ligand System?

The epidermal growth factor receptor (EGFR) plays an important role in the development and progression of prostate cancer and its overexpression is associated with decreased survival as angiogenesis proceeds in progressing prostate cancer [53]. Since WT1 represses the EGFR gene [54, 55] it could be assumed that Egr1 can stimulate this gene, and this has recently been ascertained [56]. WT1 has been found to be overexpressed in prostate cancer where it also induces the amphiregulin (Ar) gene coding for an EGFR ligand [57]. Ar plays a role in epithelial differentiation in the fetal kidney, but is associated with an early stage in mammary tumor formation in mice [58]. All of the EGF family of ligands can induce the EGFR both transcriptionally and posttranscriptionally [59, 60] and several ligands have been shown to be overproduced. For example, heparin-binding EGF (HB-EGF) is overexpressed in prostate cancer [61] and has Egr1 binding sites in its promoter, but whether Egr1 regulates this gene has not yet been tested. A role for Egr1 in prostate cancer initiation is not strongly indicated, but there is good evidence that Egr1 is involved in the maintenance of prostate tumor cell proliferation, during which, genetic mutations could drive progression.

The genes highly expressed in a prostate cancer cell line overexpressing Egr1 were analyzed using gene microarrays with the result that IGF-II, PDGF-A, and TGFβ1 were all found to be overexpressed in Egr1-expressing cells, results that were verified by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) [44]. Egr1 therefore perpetuates the growth process because some of the genes activated directly by Egr1 are growth factors themselves, such as PDGF-A and PDGF-B [62–64], FGF1 and -2 [65, 66]. Growth factor production by prostate cancer cells is thought to be important in tumor progression [67, 68]. PDGF is a major autocrine factor for prostate cancer cells [69, 70] and signals a diversity of cellular responses in vitro, including cell proliferation, survival, transformation, and chemotaxis. These genes implicate Egr1 as an important regulator of growth factor and receptor genes in prostate cancer.

The Egr1 Signaling Pathways: Egr1 protein is thought to be responsible for regulating some of the genes that are downstream effectors of growth factor stimulation of proliferation. A recent study shows that transforming growth factor alpha (TGFα) stimulated mitogenesis is mediated through the upregulation and binding of Egr1 to the cyclin D1 promoter [71]. Egr1 was shown to stimulate the thymidine kinase gene during the G_0 to G_1 transition [72] and cyclin A may also be a direct target gene for Egr1 [73]. This identifies three important target genes for Egr1 in cell cycle regulation. Ornithine decarboxylase (ODC) is repressed by WT1 [74] and is highly expressed in hyperplastic prostate tissue [75], suggesting that Egr1 may be an upregulator. Egr1 also regulates WT1, Egr2 and at least one member of a parallel zinc finger family of transcription factors, the BTEB family [11]. Egr1 autoregulates the activity of its own promoter, and there is reason to think that Egr1 may regulate the activation of c-fos and c-Jun [38], suggesting a complex regulatory system that is important for homeostasis.

Egr1 is an Angiogenic Factor

Two aspects of tumor biology have become important recently. In addition to the role of tumor produced growth factors that stimulate autocrine and paracrine growth, a major part of tumor progression is the specific stimulus to endothelial cell proliferation and angiogenesis [76] by growth factors. This stimulus is generated by hypoxia in tumor cell masses that readily become depleted of oxygen, leading to the induction of Egr1 expression. Egr1 appears to play key roles in tumor progression in a direct way in that its targets are those that may play roles in cell proliferation and survival of both prostate and endothelial cells that lead to progressive tumor development. Growth factors IGF-II, FGF2, PDGF-A, PDGF-B, EGF-like ligands and VEGF are major stimulators of endothelial cells and the development of new blood vessels that determine the progression of cancer. The expression of all of these genes is correlated with the expression of, or are direct targets of, the Egr1 transcription factor (table 1). The vascular endothelial growth factor, VEGF, is upregulated by the hypoxia transcription factor, HIF-1α, synergistically with Egr1, leading to stimulated endothelial cell growth and differentiation in tumors [77].

Endothelial cells proliferate and migrate in an environment that becomes rich in proteases and matrix molecules that stimulate cell movement and provide a migratory surface as well as structural support to new blood vessels. A subtractive cDNA analysis in prostate cell lines that selected for androgen resistance versus sensitivity, showed prominent upregulation of fibronectin (FN) expression in resistant cells [78]. FN is a known target gene for Egr1 [79]. Egr1 can also induce other matrix genes as well as proteases that degrade matrix and their inhibitors, thus restructuring matrix deposits and facilitating metastases. Hepsin is a membrane-inserted serine protease that has been found to be strongly upregulated in metastatic prostate cancer and has good potential as a therapeutic target [80–82], but it remains to be tested as a target gene of Egr1 or HIF-lα. Urokinase plasminogen activator (uPA) [83] and its receptor (uPAR), tissue inhibitors of metalloproteinases (TIMPs) [84], and several proteases such as gelatinase (MMP9) are key proteins that are induced. The pleiotropic protein that triggers the coagulation cascade, tissue factor (TF) also plays a role as the coordinator of the angiogenic and immune response signaling pathways in tumor tissues [85, 86]. This gene and others in this arena are direct or indirect target genes of Egr1 likely to play roles during tumor progression. It is interesting that TGFβ1 (a direct target of Egr1) is largely growth inhibitory to epithelial cells, but in prostate tumors TGFβ1 is considered a major factor contributing to progression, by its induction of matrix and activation of growth factors that attach to matrix for optimal effects on prostate tumor and endothelial cells.

Conclusions

Some of the known genes that are regulated by Egr1 are listed in table 1. Here, we have indicated the level of certainty that Egr1 is a direct regulator of putative target genes. The simplest indicator is a correlation of Egr1 and the putative target gene expressions measured as mRNA or protein levels after the induction of endogenous or the introduction of exogenous Egr1 expression. This includes RT-PCR-verified microarray data [44]. The second level is when the promoter of the gene is shown to be regulated by overexpression of Egr1, either exogenous or induced, using a promoter-reporter construct. This is usually supported by DNA-binding studies using techniques such as electrophoretic mobility shift assays (EMSA). The best way to prove that a gene is a *direct* target of Egr1 is to show that the promoter of the target gene can be recovered from genomic DNA in the cell as a cross-linked chromatin fragment after immunoprecipitation with an Egr1-specific antibody. Only a few genes have been verified as target genes of any transcription factor by this criterion and for

Egr1 they include TGBβ1 [87], TF, TNFα [I. de Belle, M. Guha and E. Adamson, unpubl. obs.] and PTEN [141] and a newly isolated and characterized gene called TOE1 [de Belle et al., in preparation].

This review has focussed on the role of Egr1 in prostate cancer because the expression of this transcription factor is constitutively elevated in prostate tumors. Although there is evidence pointing to an important role for Egr1 in tumor progression in the prostate, the mechanisms for the multiple effects of Egr1 in cellular responses to stimuli are not well worked out. Some key genes such as IGF-II, PDGF-A, VEGF, TGFβ1 and FN are likely directly regulated by Egr1, but how most of these regulated genes induce different cellular responses in different cell types remains to be discovered.

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In Vivo Cloning and Characterization of a New Growth Suppressor Protein TOE1 as a Direct Target Gene of Egr1*

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Egr1, an immediate early transcription factor, responds to diverse stimuli and affects gene transcription to accomplish its biological effects. One important effect of Egr1 expression is to decrease the growth and tumorigenic potential of several tumor cell types. To identify important Egr1 target genes, we have adapted a methodology involving formaldehyde-induced protein-DNA cross-linking, chromatin immunoprecipitation, and multiplex PCR. Using this approach, we report the cloning of a new Egr1 target gene that is able to account, at least in part, for the growth inhibitory activity of Egr1. We have named this new protein TOE1 for target of *Egr1*.

A common feature associated with the expression of immediate-early genes is their rapid, transient response to a diverse variety of extracellular signals. We have been studying the properties of the early growth response gene, Egr1, which can be transcriptionally induced by a wide spectrum of stimuli including growth factors, cytokines, stresses, depolarizing stimuli, phorbol esters, vascular injury, and irradiation, both ionizing and nonionizing, in a rapid and transient manner with kinetics mirroring those of c-*fos* (1). We have previously presented evidence suggesting a role for Egr1 in suppressing tumor cell growth (2, 3). Specifically, we demonstrated that overexpression of Egr1 in transformed cells suppresses growth in soft agar as well as inhibits their tumor formation in nude mice. Furthermore, it was shown that the DNA-binding domain of Egr1 is necessary for its ability to suppress tumor formation, highlighting the importance of its transactivation of downstream genes in this process (4). Together these results indicate that transformed cells can be induced to revert to normal growth patterns following the re-expression of Egr1. These studies suggest that the loss of Egr1 may result in the loss of cellular homeostasis because of a deficit in Egr1-responsive genes and that this may play a pivotal role in tumorigenesis. Clearly, the identification of a genetic profile of Egr1-responsive genes would constitute a significant step in understanding the different activities associated with Egr1, including its role in cellular growth control. Over the past several years there have been numerous studies identifying various individual Egr1 target genes in diverse cell and tissue types. Reported Egr1 targets include TGF-β1,[1] platelet-derived growth factors A and B, basic fibroblast growth factor, tissue factor, interleukin 2, and CD44 to mention only a few (reviewed in Ref. 5). These studies have focused on the *in vitro* analysis of an individual target gene in a specific cell type under a defined set of experimental conditions. As a step toward a more complete understanding of the biological role for a transcription factor, it would be informative to be able to identify *in vivo* target genes.

Currently, few techniques are available to address this issue. Both differential display and subtractive hybridization analyses are aimed at isolating messages that are up- or down-regulated from pools of RNA isolated from cells or tissues either positive or negative for the gene in question. One clear drawback with both of these techniques is that they select for any RNA message that shows a change in expression pattern. Therefore, when screening for changes in gene expression induced by a transcription factor, these methods do not select purely for direct targets. Recently we and others have described a method for the direct isolation of protein-bound DNA involving *in vivo* chemical cross-linking using formaldehyde followed by immunoprecipitation from chromatin (ChIP). This method was successfully used in applications ranging from examining chromatin structures surrounding the polycomb group proteins during *Drosophila* development (6) and the identification of nuclear matrix attachment sites (7) to the isolation of DNA sequences bound by Egr1 (8). In addition, the same cross-linking method has been used to examine nucleosomal structure, transcription factor occupancy of promoter sites, regions of histone acetylation, and mapping of telomere silencing protein binding, illustrating its broad application utility (9–12). Recently, coupling the ChIP approach with hybridization to genomic or promoter region DNA microarrays has allowed a comprehensive characterization of *in vivo* transcription factor DNA binding patterns (13–16).

In this report we have extended ChIP technology, allowing gene discovery of Egr1 target genes by multiplex PCR. Moreover, we present the cloning of a newly identified gene, called *TOE1*, as an Egr1 target gene. We have characterized TOE1 as a cell growth inhibitor by altering the cell cycle through the induction of p21. Furthermore, we show that the increase in the p21 level is consistent with a mechanism involving TGF-β1.

MATERIALS AND METHODS

Cells, Transfection, Antibodies, and Growth Assays—Both the H4 clone derived from the human fibrosarcoma cell line HT1080 and the

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Ian de Belle and Sabina Sperandio dedicate this manuscript to the memory of Ted and Marilyn Crain.

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[1] The abbreviations used are: TGF, transforming growth factor; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase.

Egr1 stably transfected H4 subclone E9 have been previously described (4). 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All of the DNA transfections were performed using LipofectAMINE 2000 (Invitrogen), following the manufacturer's instructions. Antibodies against cdc2, phospho-cdc2(Y15), and phospho-p53(S15) were from Cell Signaling Technology. Antibodies against cyclin B1, p21, and p53 were from Santa Cruz Biotechnology. Anti-actinin and the M2 monoclonal anti-FLAG antibody were from Sigma. For cell growth assays 20×10^3 control and TOE1 expressing 293 selected and pooled clones were seeded into 96-well plates in triplicate. At the indicated times, cell growth was determined using the CellTiter Cell Proliferation Assay (Promega).

In Vivo Formaldehyde Cross-linking and Chromatin Immunoprecipitation—Cross-linking and chromatin immunoprecipitation were performed as previously described (6, 8). Briefly, the cells were grown in 150-mm plates to 80–90% confluence and then cross-linked by the addition of buffered formaldehyde to a final concentration of 1%. Following exposure to formaldehyde at room temperature for a period of 30 min, the cells were lysed by sonication and chromatin purified by centrifugation through a 5–8 M urea gradient in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). Purified chromatin was dialyzed against 10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5% glycerol to remove the urea. Samples of 30–60 μg of chromatin were digested with 10 units of *Eco*RI overnight at 37 °C and then precleared by the addition of nonimmune rabbit serum and protein A-Sepharose beads. The precleared samples were immunoprecipitated with affinity purified anti-Egr1 antibodies and protein A-Sepharose beads (17). DNA fragments cross-linked and co-precipitating with Egr1 were purified and ligated to *Eco*RI linkers consisting of 5′-AATTCGAAGCTTGGATCCGAGCAG-3′ and 5′-CTGCTCGGATCCAAGCTTCG-3′. Following ligation, the products were amplified using the 20-mer as primer. Amplification conditions were 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 4 min for 30 cycles. For direct amplification of the ChIP samples, no linker ligation was performed, and direct amplification from the Egr1 immunoprecipitates was done using specific primers for TOE1 (see below), TGF-β, and cyclophilin. The TGF-β primers used were 5′-GGGCTGAAGGGACCCCCCTC-3′ and 5′-TCCTCGGCGACTCCTTCCTC-3′. The cyclophilin primers used were 5′-CTCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′.

Library Multiplex PCR and TOE1 cDNA Cloning—Following amplification of linker-ligated products as described above, the linkers were removed by *Eco*RI digestion, and the products were purified using a PCR product purification kit (Roche Molecular Biochemicals). Multiplex PCR was performed using 100 ng of PCR products as the 5′ primer mix and a T7 oligonucleotide as the 3′ primer, with 100 ng of an excised undifferentiated NT2 cell cDNA library (Stratagene). 30 cycles of hot start PCR were performed using the following parameters: 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 4 min. A 2-kilobase pair band derived from the multiplex PCR was excised from the gel, eluted, cloned into the pCR3.1 TA cloning vector (Invitrogen), and sequenced. Data base homology searches were performed using the BLAST program. To confirm the full-length TOE1 cDNA, we performed 5′ rapid amplification of cDNA ends using the fetal brain Marathon-Ready cDNA kit (Clontech), following the manufacturer's instructions. The TOE1 specific primer used for 5′ rapid amplification of cDNA ends was 5′-GTGAGGGGTACAGCTTTGCC-3′. A FLAG-tagged TOE1 expression vector was generated by PCR using the following primers: 5′-CCGAAGCTTATGGATTACAAGGACGACGACGATAAGGCCGCCGACAGTGAC-3′ incorporating the FLAG epitope tag and 5′-CCGGAATTCTCAGCTACTGCCCCAA-3′. PCR was performed for 30 cycles of 95 °C for 45 s, 62 °C for 30 s, and 72 °C for 2 min. The PCR product was digested with *Hin*dIII/*Eco*RI and cloned into the same sites in pcDNA3. All of the constructs were sequence-confirmed.

Cloning of the TOE1 Proximal Promoter and Luciferase Assays—The proximal region of the TOE1 cDNA sequence was cloned from human genomic DNA using the Advantage-GC genomic PCR kit (Clontech). Primers used for PCR were 5′-GCCGGTACCCGCTCTTACACC-3′ and 5′-CCCGTTAACGACACCGCTCGT-3′. The PCR parameters used were 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min for a total of 30 cycles. This reaction generated a 580-bp product immediately 5′ of the initiation codon. The PCR product was digested with *Kpn*I and *Hpa*I and cloned into the *Kpn*I and *Sma*I sites of pGL3basic (Promega). 293 cells were transfected in 12-well plates with a total of 500 ng of DNA using LipofectAMINE 2000 (Invitrogen). Transfected DNA consisted of 200 ng of expression vector DNA, 200 ng of reporter DNA, and 100 ng of cytomegalovirus-β-galactosidase DNA for normalization. 24 h after transfection, the luciferase assays were performed as described (8).

Mutagenesis—To generate the TOE1 expression construct without the putative nuclear localization signal, QuikChange mutagenesis (Stratagene) was performed. The primers used were 5′-GCGGCAGAGGACGCTTTATTGAACCTA-3′ and 5′-TAGGTTCAATAAAGCGTCCTCTGCCGC-3′. Construction of the correct deletion was confirmed by sequencing.

Gel Shift—The gel shift assay was performed as previously described (8) using the 580-bp radiolabeled TOE1 promoter region described above and recombinant Egr1 protein.

Confocal Microscopy—Control and TOE1 expressing H4 cells were dually stained with rabbit anti-FLAG (Affinity Bioreagents) and mouse anti-nucleolin (Santa Cruz Biotechnology) antibodies. Secondary labeling was performed using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) and Texas Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch).

Flow Cytometry—The cells were harvested and fixed in 70% methanol and stored at −20 °C until all of the samples were collected. The cells were collected by centrifugation at $2000 \times g$ for 3 min, and the cell pellets were suspended in phosphate-buffered saline, digested with RNase A, and stained with propidium iodide.

Northern Blotting—A human Multiple Tissue Northern blot (Clontech) was hybridized with a PCR-generated TOE1-specific ^{32}P-labeled probe using the primers 5′-AAGCGGCGACGGCGACGACG-3′ and 5′-GTGAGGGGTACAGCTTTGCC-3′ following the manufacturer's instructions.

RT-PCR—To detect TOE1 expression following Egr1 transfection, total RNA was harvested from transfected cells using Tri Reagent (Molecular Research Center). Following DNase I treatment, 2 μg of RNA was used for reverse transcription using Moloney murine leukemia virus reverse transcriptase (New England Biolabs). TOE1 expression was then assessed by PCR using the same primers described above for Northern probe preparation, and glyceraldehyde-3-phosphate dehydrogenase expression was determined as a loading control using the primers 5′-AACCATGAGAAGTATGACAAC-3′ and 5′-GTCATACCAGGAAATGAGCT-3′. Expression of the p21 gene was determined using the primers 5′-CTCAAATCGTCCAGCGACCTT-3′ and 5′-ACAGTCTAGGTGGAGAAACGGGA-3′. TGF-β1 expression was assessed using the primers 5′-GCCCTGGACACCAACTATTGCT-3′ and 5′-AGGCTCCAAATGTAGGGGCAGG-3′, and cyclophilin A was amplified using the primers 5′-CTCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′. PCR conditions were 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min for 25 cycles.

Real time PCR reactions were performed using the one-step RT-PCR SYBR green kit from Roche using a Roche Light Cycler instrument. Following the RT reaction for 30 min, the PCR conditions were 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s for 40 cycles. mRNA quantitation was performed by measuring cyclophilin mRNA levels against a standard curve measurement of cyclophilin mRNA from a control sample. The primers used are described above.

In Vitro Kinase Assay—*In vitro* phosphorylation was performed as described (18).

RESULTS

Cloning of TOE1—We have previously characterized a clone of HT1080 cells, called H4, as a cell line that does not express either basal or UV-induced Egr-1. We have also described a series of stable transfected Egr1 clones (19). We used the clone with the maximum expression of Egr1, termed E9, to isolate and identify *in vivo* Egr1 target genes. We performed formaldehyde cross-linking on untreated and UV-stimulated cells followed by chromatin immunoprecipitation as described earlier (8). Because it is generally accepted that Egr1-binding sites usually occur within the proximal promoter region of genes, our immunocaptured Egr1-bound sequences are likely to consist of predominantly promoter regions with extensions into the 5′-untranslated region and even into the coding region. To identify target gene sequences we performed multiplex PCR using our immunocaptured Egr1-bound DNA sequences as 5′ multiplex primers. As template we selected a cDNA library and used a T7 primer that anneals 3′ to all cDNAs permitting full-length cDNA amplification. Using DNA captured from E9 cell Egr1 immunoprecipitates, we found that multiplex PCR-amplified products only in the presence of the multiplex primers, cDNA library, and the 3′ T7 primer (Fig. 1*A*, *lane 2*). When multiplex primers derived from UV-treated E9 cells were used, on occa-

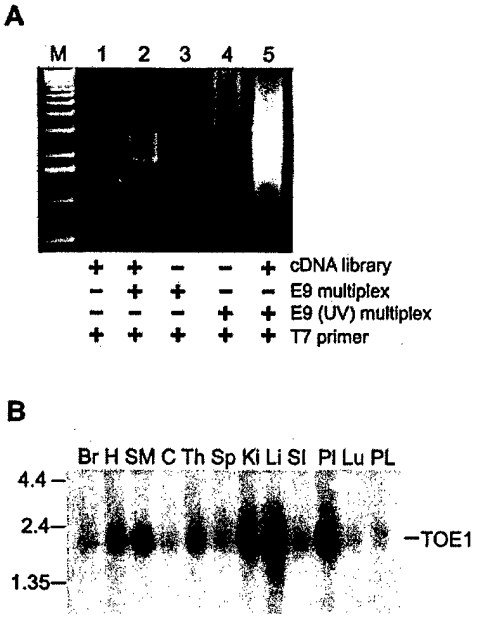

FIG. 1. **Cloning and characterization of the newly identified Egr1 target gene *TOE1*.** *A*, multiplex PCR amplification of Egr1 target genes from a NT2 cDNA library. Linker PCR amplification products of DNA from Egr1 containing immunoprecipitates were used as multiplex primers in a PCR reaction containing a NT2 cell cDNA library template as well as the T7 3′ primer. cDNA amplification products are seen in *lanes 2* and *5*, where all components are present. Both control and UV-treated E9 cells produced PCR products. *Lane M*, 1-kilobase pair DNA markers. *B*, multiple tissue Northern blot hybridized with a *TOE1* probe shows expression of an approximate 2-kb message in adult human tissues. The nucleotide sizes are indicated to the *left*. *Br*, brain; *H*, heart; *Sm*, skeletal muscle; *C*, colon; *Th*, thymus; *Sp*, spleen; *Ki*, kidney; *Li*, liver; *SI*, small intestine; *Pl*, placenta; *Lu*, lung; *PL*, peripheral leukocytes.

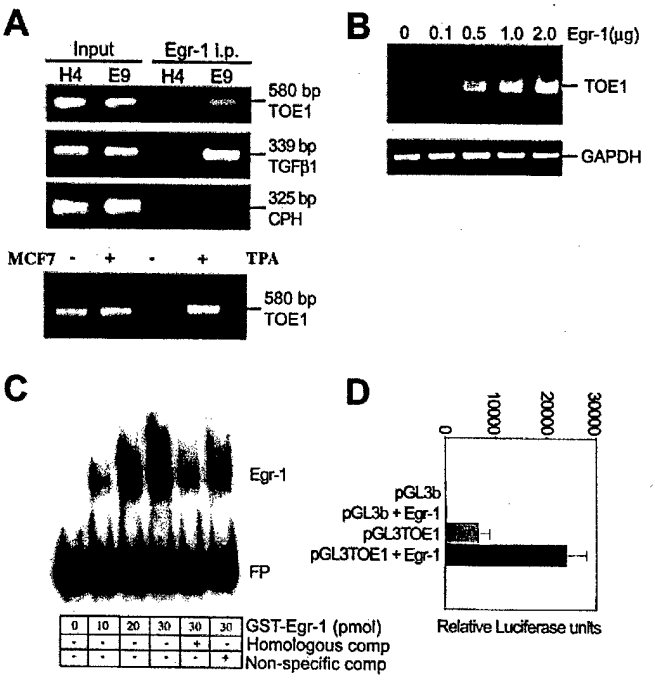

FIG. 2. **Egr1 binds to the 5′ region of *TOE1* and activates its expression.** *A*, PCR amplification of the *TOE1* 5′ region from cross-linked chromatin. Either total cross-linked chromatin (*Input*) or Egr1 immunoprecipitates (*Egr1 i.p.*) were screened directly for the presence of *TOE1* 5′ sequences by PCR using primers designed to amplify a 580-bp fragment 5′ of the initiation codon. The same samples were also used for amplifications using primers for TGF-β1 and cyclophilin A. The same primers were used to analyze Egr1 immunoprecipitates from untreated or 12-*O*-tetradecanoylphorbol-13-acetate-treated MCF7 cells. *B*, Egr1 expression activates *TOE1* expression. RT-PCR amplification of TOE1 from Egr1 transfected H4 cells. Increasing amounts of Egr1 (shown above the lane) were transfected into H4 cells, and total RNA was prepared 24 h later to perform RT-PCR for *TOE1*. Primers within the coding sequence of *TOE1* were designed to amplify a 454-bp product. An equal RNA loading in the RT-PCR reaction was determined using primers amplifying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *C*, Egr1 binds directly to the TOE1 5′ region. The 580-bp region upstream of the initiation codon of *TOE1* was used as a probe in the gel shift assay. Increasing amounts of recombinant Egr1 showed the binding to this region. Specific binding was determined by adding either unlabeled homologous probe DNA or nonspecific DNA at a 50-fold molar excess. The positions of the free probe (*FP*) and Egr1 shift (*Egr1*) are indicated. *D*, Egr1 transactivates expression from the *TOE1* 5′ region. The same 580-bp 5′ sequence from TOE1 was cloned into the pGL3basic luciferase reporter. Empty reporter vector or the TOE1 reporter in the presence or absence of co-transfected Egr1 expression vector were transfected into 293 cells. 24 h later the cells were harvested and analyzed for luciferase activity. The results have been normalized for transfection efficiency as determined by β-galactosidase measurements. The results are plotted as the average values ± standard deviations. The experiment was repeated three times with similar results.

sion we found some self-amplification from the multiplex primers resulting in a high molecular weight smear (Fig. 1*A*, *lane 4*). However, the addition of cDNA library template produced a much stronger and distinctly different profile of amplified products (Fig. 1*A*, *lane 5*), suggesting that cDNAs were obtained from these primers as well. To directly address the question of whether these amplified cDNAs represented *bona fide Egr1* target genes, we isolated and cloned an individual target gene.

We focused on the distinct DNA band amplified using primers isolated from E9 cells and migrating with an approximate size of 2 kb (Fig. 1*A*, *lane 2*). Cloning and sequencing of this DNA revealed an open reading frame coding for a predicted polypeptide of 510 amino acids and with a predicted molecular mass of ~58 kDa. To confirm that this clone represented a full-length cDNA, we performed 5′ rapid amplification of cDNA ends. Sequencing results confirmed that the captured sequence represented a full-length cDNA clone. A data base homology search of the DNA sequence identified the chromosomal map position on human chromosome 1 (1p34.1–35.3). Comparison of the sequence of this region of chromosome 1 to our cloned cDNA identified an 8 exon gene. BLAST homology searches (20) revealed no extended homology with any known protein. However, a potential single zinc finger was noted as well as a possible nuclear localization signal.

To show that the clone represented an expressed gene, a multiple tissue Northern blot was hybridized and showed intense hybridization to a 2-kb mRNA species in six of the 12 tissues with the highest level of expression in placenta, liver, and kidney (Fig. 1*B*). We cloned the open reading frame of the cDNA, together with a FLAG epitope tag, into a mammalian expression vector and transfected the construct into H4 cells. Western analysis of cells transfected with the FLAG-tagged expression vector and anti-FLAG antibodies showed that the expressed protein migrated on SDS-PAGE with a molecular mass of ~60 kDa, in close agreement with its predicted mass of 58 kDa (data not shown).

TOE1 Is a Target for Egr1 Binding and Transactivation—To confirm the specificity of Egr1 binding to *TOE1 in vivo*, DNA recovered from immunoprecipitates was PCR-amplified to detect the 5′ region of *TOE1*. As shown in Fig. 2*A* we were able to amplify *TOE1* from E9 but not from H4 immunoprecipitates. We did, however, confirm the presence of the *TOE1* gene in the total chromatin fraction, thus ruling out the formal possibility that the *TOE1* gene is deleted in H4 cells. Further, the known Egr1 target gene TGF-β was also amplified from E9 cells (21). The lack of amplification of cyclophilin sequence served as a negative control. This provided evidence that *TOE1* was indeed a target of Egr1 in these cells and that the immunoprecipitated

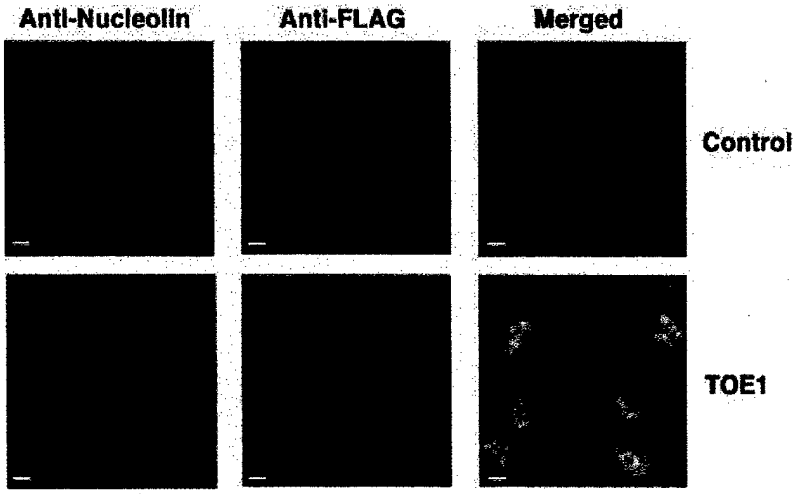

FIG. 3. **TOE1 is a nuclear/nucleolar protein.** Control vector and a FLAG-tagged *TOE1* expression vector were transfected into H4 cells. The cells were immunostained with antibodies to FLAG and to the nucleolar protein nucleolin. Texas Red and fluorescein isothiocyanate-labeled secondary antibodies were used to label nucleolin and FLAG, respectively. Confocal microscopy was performed showing nucleolar co-localization of TOE1 and nucleolin. The *bar* in each *panel* represents 10 microns.

DNA included the 5′ region of the gene. Because E9 cells constitutively overexpress Egr1, we sought to determine whether TOE1 is an Egr1 target in an alternate cell type upon transient Egr1 induction. MCF7 cells were stimulated to express Egr1 by 12-*O*-tetradecanoylphorbol-13-acetate treatment, and then the ChIP assay was performed on untreated or 12-*O*-tetradecanoylphorbol-13-acetate-treated cells. The results shown in Fig. 2*A*, TOE1 was also an Egr1 target gene in these cells. To determine the role of Egr1 in regulating the transcription of *TOE1*, we used RT-PCR following transfection with an increasing amount of an Egr1 expression vector and found a proportional increase in TOE1 expression (Fig. 2*B*).

Direct binding of Egr1 to the *TOE1* promoter region was assessed by a gel shift analysis using as probe a region spanning 580 bp upstream of the translation start. Using recombinant Egr1 we found specific binding to the probe (Fig. 2*C*). When oligonucleotides representing the consensus Egr1-binding site were used as competitor, effective competition was also observed (data not shown). As a test of the functional properties of the complex we inserted the same 580-bp 5′ region upstream of a luciferase reporter. We observed that this region responds to Egr1 expression by activating transcription (Fig. 2*D*). Together, these results are consistent with *in vivo* binding of Egr1 to and transactivation of the *TOE1* gene.

Subcellular Localization of TOE1—To determine the intracellular localization of TOE1, a FLAG-tagged expression construct was transfected into H4 cells. As shown in Fig. 3, following immunostaining for the FLAG epitope, the subcellular localization of TOE1 was distinctly nuclear. Transfection and staining of H4 and 293 cells (not shown) showed patterns of concentrated localization within the nucleus. These sites of concentration appeared to correspond to nucleoli. Dual staining using anti-FLAG and anti-nucleolin antibodies followed by confocal microscopy (Fig. 3) showed that most of the expressed TOE1 co-localized with nucleolin, indicating a predominant nucleolar location for TOE1. In addition to its nucleolar localization we observed intense staining for TOE1 as multiple nuclear speckles. As noted above, data base homology searches identified a putative nuclear localization sequence consisting of KRRRRRRREKRKR located at positions 335–347 in the 510-amino acid protein. Deleting the putative nuclear localization basic stretch of amino acids resulted in the cytoplasmic localization of TOE1 (Fig. 4), suggesting that this sequence is responsible for TOE1 nuclear targeting.

TOE1 Expression Affects the Growth of 293 and H4 Cells—To test whether TOE1 might be involved in mediating the growth effects of Egr1, we measured the growth rate of cells stably transfected with a TOE1 expression vector. Fig. 5*A* shows that

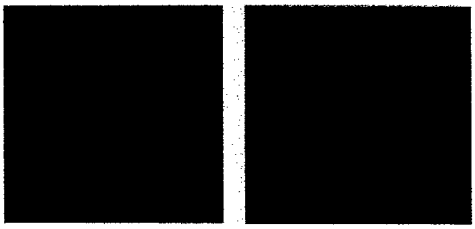

FIG. 4. **Identification of TOE1 nuclear localization sequence.** H4 cells were transfected with either a FLAG-tagged wild type TOE1 expression vector (*left panel*) or a FLAG-tagged TOE1 expression vector containing a deletion in the putative nuclear localization sequence (*right panel*). Following fixation, the cells were subjected to immunostaining using anti-FLAG (*red*). For the cells expressing the TOE1 nuclear localization sequence deletion, the nuclei were counterstained with 4′,6-diamidino-2-phenyl.

the growth rate of TOE1-expressing cells was severely reduced in comparison with empty vector control cells. The doubling time for control cells was ~24 h, whereas a pool of TOE1 expressing clones required 40 h to double in number. Transfection of the same vector expressing the calcium binding protein calbindin had no effect on cell growth (data not shown), suggesting that inhibition by TOE1 was not a nonspecific effect of protein over expression. Similar results were obtained in H4 cells (data not shown).

Cell growth inhibition in TOE1-expressing cells was also examined by performing colony forming assays. Control cells formed numerous rapidly growing colonies, whereas TOE1-expressing cells were only able to form 30% as many colonies (data not shown). To determine whether the decrease in cell growth of TOE1-expressing cells represented a generalized slowing of growth or a cell cycle stage-specific slowing, we performed flow cytometry on log phase cells. We found a significant increase in the fraction of cells present in the G_2/M phases of the cell cycle in TOE1-expressing cells (27%), compared with the control cells, with 13% of the cells in this fraction (Fig. 5*B*). We found no difference between the mitotic index of control and TOE1-expressing cells, suggesting that TOE1 was pausing the cells in the G_2 phase (data not shown). In addition, it should be noted that we found TOE1 expression to be highly influenced by the growth state of the cells. Specifically, we have found TOE1 expression to be regulated by cell culture density, possibly indicating a form of activation caused by contact inhibition.[2] The expression of TOE1 in dense cell cultures occurred even in cells that cannot express Egr1, indicating that although Egr1 can activate expression of TOE1, the

[2] I. de Belle and J.-X. Wu, unpublished observation.

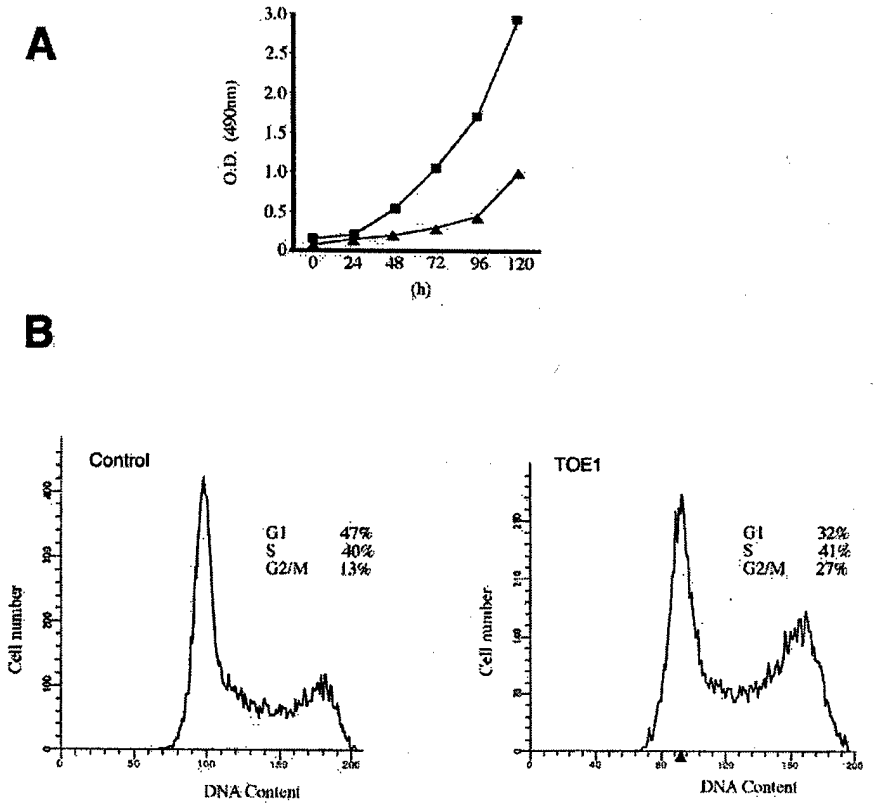

FIG. 5. **TOE1 expression affects cell growth and the cell cycle.** *A*, TOE1 decreases the growth rate of 293 cells. Pooled clones of empty vector or *TOE1*-expressing cells were used to determine their growth rate over a period of 5 days. *Solid squares*, control transfected cells; *solid triangles*, *TOE1*-expressing cells. The results are the averages of triplicate readings, and the experiment was repeated three times with similar results. *B*, *TOE1* expression affects the cell cycle. The cell cycle distribution of log phase growing control and TOE1 expressing clones of H4 cells was determined by flow cytometry. The calculated percentages of the cell cycle phases are indicated.

gene must be subject to additional forms of regulation.

TOE1 Causes an Increase in p21 Expression in H4 Cells—To investigate the mechanism of TOE1 induced G_2 phase delay, we performed Western blotting on several G_2 cell cycle markers. Fig. 6*A* shows that there was no significant change in cyclin B1, cdc2, or phospho-cdc2 levels between control, TOE1, and mutant TOE1-expressing cells (with the nuclear localization deleted). This suggested that the activation potential of the G_2-specific CDK complex was unaffected by the expression of TOE1. We therefore examined the possibility that the activity of the complex might be modulated by its known inhibitor p21. The level of p21 was dramatically up-regulated in TOE1-expressing cells but not in either control or TOE1 mutant cells. Because p53 is a known transactivator of the p21 gene, we examined the level and activation of p53 in our cells. We were unable to find a significant induction or activation of p53, at least insofar as serine 15 phosphorylation is concerned. Further exploration of the induction of p21 using RT-PCR showed that TOE1-expressing cells up-regulated p21 at the mRNA level (Fig. 6*B*). This activation was not seen in cells expressing non-nuclear mutant TOE1. To demonstrate that the increase in p21 was functionally associated with an effect on cdc2 activity, we immunoprecipitated cyclin B1 and measured the associated kinase activity *in vitro* with histone H1 as substrate. Fig. 6*C* shows a significant decrease in kinase activity only in TOE1-expressing cells, correlating with increased p21 expression in those cells.

Increased TGF-β1 in TOE1-expressing cells—Because Egr1 expression is known to affect TGF-β1 levels (21), we sought to determine whether the increase in p21 levels might be mediated by TGF-β1. Using real time quantitative PCR, we examined the TGF-β1 levels in cells transfected with a TOE1 expression vector. As shown in Fig. 7, using both MCF7 and H4 cells lines, we noted an increase in the level of TGF-β1 mRNA in TOE1 transfected cells compared with control transfected cells.

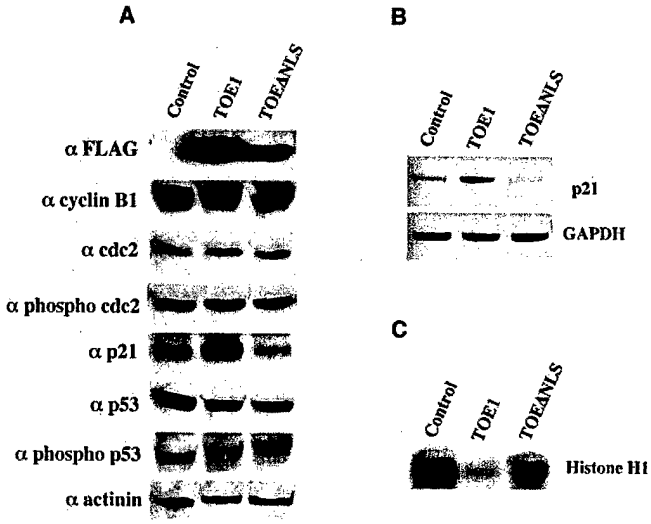

FIG. 6. **TOE1 affects growth inhibition through increased p21 expression.** *A*, control, TOE1, and TOE1ΔNLS cells were probed by Western blotting with the indicated antibodies. *B*, RNA was extracted from cells, and RT-PCR was performed for the expression of p21 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *C*, *in vitro* phosphorylation assay. Cyclin B1 immunoprecipitates were incubated with histone H1 and radiolabeled ATP. The products were visualized by SDS-PAGE and autoradiography.

DISCUSSION

With these studies we report, for the first time, the application of chromatin immunoprecipitation to cDNA cloning using a form of multiplex PCR. We have demonstrated that this technique was successful not only in cloning transcription factor target genes but also in the identification of a new target for Egr1. Together our results indicated that the multiplex amplification produced a genuine cDNA and that the cloned DNA represented an expressed gene. This newly cloned gene encodes

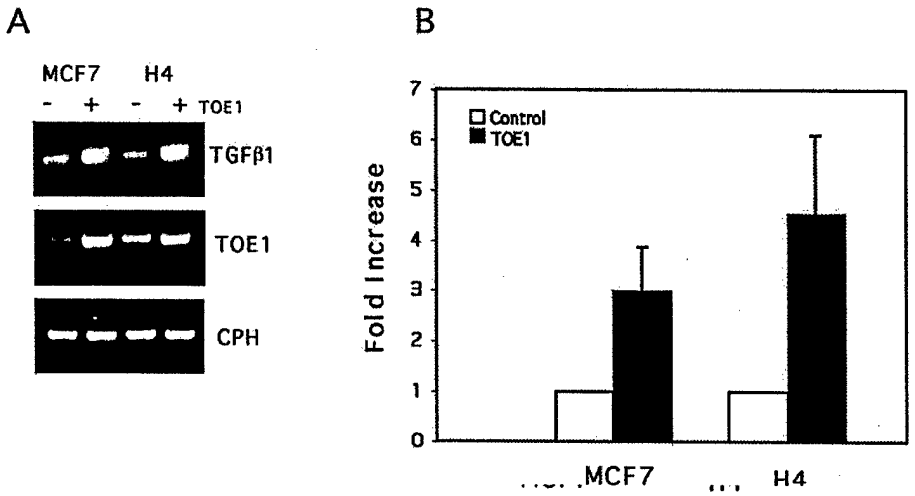

FIG. 7. **TOE1 expression affects the levels of TGF-β1 mRNA.** *A*, RT-PCR measurement of TGF-β1 and TOE1 from MCF7 and H4 cells transfected with an empty vector control (−) or a TOE1 expression vector (+). 24 h after transfection, RNA was harvested from the cells, and RT-PCR was performed using the protocol described under "Materials and Methods" with 25 cycles of amplification. Cyclophilin A amplification was used to demonstrate the equal RNA amounts included in each reaction. *B*, real time quantitative PCR was performed on MCF7 and H4 cells transfected with either control empty vector or a TOE1 expression vector. 24 h following transfection, RNA was collected and subjected to real time PCR for TGF-β1 mRNA quantitation. The *open bars* represent the relative quantity of TGF-β1 level in control cells, and the *closed bars* represent that for TOE1 transfected cells. mRNA samples were normalized to cyclophilin A levels. The results shown are the averages of four independent experiments showing standard deviations.

a 510-amino acid protein that we have shown to be an authentic Egr1 target gene. To confirm that the gene codes for an endogenously expressed protein, we have recently raised a polyclonal antibody using a synthetic peptide epitope derived from the predicted amino acid sequence. Preliminary testing has shown reactivity against both recombinant and an endogenous protein of identical molecular mass, suggesting that the cDNA is expressed at both the mRNA and protein level.

During the course of these studies an unpublished and unnamed cDNA generated through a library sequencing effort was deposited in the GenBank™ data base that was identical to our cloned cDNA (nucleotide accession number AK024011). Based on the sum of our observations, we have called this cDNA the HUGO approved name and symbol *TOE1* for target of *Egr1*. Expression of TOE1 was detected in all of the adult human tissues examined but at varying levels, indicating that the regulation of this gene may vary depending on cell or tissue type.

Examination of the sequence of TOE1 did not reveal conserved domain structures apart from a single potential zinc finger and a possible nuclear localization signal. Immunostaining confirmed that TOE1 was found localized to the nucleoplasm and nucleolus. Despite the absence of a recognized DNA-binding domain, we have examined the possibility that TOE1 might participate in transcriptional regulation. However, TOE1 cloned as a GAL4 fusion failed to activate a GAL4-binding site reporter, suggesting that TOE1 alone is not sufficient for transcriptional regulation. The possibility remains that TOE1 can participate in transcriptional regulation through protein interactions and indirect DNA association not recapitulated in the GAL4 fusion experiments. Although no extended homology to any known gene was noted by BLAST searches, a limited region of homology to poly(A)-specific deadenylation nuclease was revealed. We are currently investigating the possibility that TOE1 may function as a nuclease.

To better understand the biological role of TOE1, we examined the effects of its expression and noted a dramatic decrease in both the growth rate and colony growth of H4 cells. We found that this was not the result of a general decrease in growth rate but rather was due to a G_2 cell cycle phase delay. Furthermore, the G_2-specific cell cycle delay correlated with an increase in the expression of the cyclin-dependent kinase inhibitor p21. Deletion of the nuclear localization signal abrogated this effect, suggesting not only that TOE1 could induce cell cycle-specific G_2 pausing but also that its nuclear/nucleolar localization was critical for this function. The localization of TOE1 in the nucleolus may provide further evidence for a role in cell cycle regulation because it has been found that many important cell cycle proteins can be found in the nucleolus as a means of sequestration, thereby limiting their function until the appropriate time (22–24).

Because p21 is also able to inhibit cyclin-dependent kinase activities controlling passage through the G_1 restriction point, it would be predicted that the TOE1-directed increase in p21 levels would also display a G_1 phase pausing. Although we did not see this in log phase growing cells, when cells were synchronized in the M phase and then released to pass through G_1, we noted a marked delay in the TOE1-expressing cells (data not shown). This suggested that the increase in p21 levels was also active at the G_1 check point, but this was only seen if cells had been synchronized outside of the G_2 phase. Although p21 is well known for its activity in G_1 phase pausing, its role in G_2 is being increasingly recognized (25, 26). These results suggest that the mechanism by which TOE1 affects cell growth is through transcriptional up-regulation of the p21 gene. We have not, however, formally ruled out the possibility that the increase in p21 levels might be due to an increase in transcript stability rather than increased expression. Also, we have not completely ruled out a contributing role for p53 in the up-regulation of p21 but have demonstrated that p53 levels and serine 15 phosphorylation were not altered. Further, we have provided evidence that TOE1-dependent TGF-β1 activation may participate in the increase in p21. However, it also remains possible that TOE1 and p53 cooperate in the transactivation of p21 either directly or indirectly. We have preliminary evidence that TOE1 and p53 are able to interact physically, but the significance and specificity of this interaction remain to be analyzed.[3] Although the precise mechanism of action remains to be studied, our results have shown that expression of TOE1

[3] I. de Belle, unpublished observation.

leads to growth inhibition as well as a decrease in colony forming ability, likely involving the activation of p21. Given that these same features are seen following expression of Egr1, we expect that the downstream target TOE1 plays an important role in executing this physiological function of Egr1 in its proposed role as a tumor suppressor.

Finally, It is intriguing to note that the chromosomal location of *TOE1* maps to 1p34.1–35.3. Deletion of the distal portion of 1p accounts for a significant proportion of chromosome 1 aberrations and has been observed in brain, breast, ovarian, colorectal, and other tumor types (27–29). Combined data suggest that chromosome 1p likely harbors one and possibly multiple tumor suppressor genes, and given the growth inhibitory effect of TOE1, we are currently investigating the possibility that TOE1 may also function in this capacity.

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Egr1 Promotes Growth and Survival of Prostate Cancer Cells

IDENTIFICATION OF NOVEL Egr1 TARGET GENES*

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In the majority of aggressive tumorigenic prostate cancer cells, the transcription factor Egr1 is overexpressed. We provide new insights of Egr1 involvement in proliferation and survival of TRAMP C2 prostate cancer cells by the identification of several new target genes controlling growth, cell cycle progression, and apoptosis such as cyclin D2, P19ink4d, and Fas. Egr1 regulation of these genes, identified by Affymetrix microarray, was confirmed by real-time PCR, immunoblot, and chromatin immunoprecipitation assays. Furthermore we also showed that Egr1 is responsible for cyclin D2 overexpression in tumorigenic DU145 human prostate cells. The regulation of these genes by Egr1 was demonstrated using Egr1 antisense oligonucleotides that further implicated Egr1 in resistance to apoptotic signals. One mechanism was illustrated by the ability of Egr1 to inhibit CD95 (Fas/Apo) expression, leading to insensitivity to FasL. The results provide a mechanistic basis for the oncogenic role of Egr1 in TRAMP C2 prostate cancer cells.

Prostate cancer is the most common malignancy in men and a frequent cause of cancer death. The mortality of this disease is due to metastasis to the bone and lymph nodes. Prostate cancer progression is thought to proceed from multiple defined steps through prostatic intra-epithelial neoplasia, invasive cancer, and progression to androgen-independent and refractory terminal phase (44, 50). A large fraction of early onset, and up to 5–10% of all prostate cancer patients, may have an inherited germline mutation that has facilitated the onset of carcinogenesis. However, in the majority of cases, no inherited gene defects are involved, and cancer arises as a result of a series of acquired somatic genetic changes affecting many genes on several chromosomes. Although the molecular mechanism of prostate cancer progression remains largely unknown, a few genes such as E-cadherin, α-catenin, TGF-β,[1] and insulin-like growth factors I and II have been shown to be aberrantly expressed and are markers of prostate cancer (34, 69). To clearly understand the multistep progression of this disease many other genes remain to be identified.

One of the overexpressed genes found in prostate cancer tissue is the transcription factor early growth response gene 1 (Egr1) (18, 62). This gene could have an important function because its expression level increases with the degree of malignancy as measured by the Gleason grade of the tumor (18). This seems to be specific to prostate tumor cells, because in mammary and lung tumors, as well as most normal tissues, Egr1 expression is low. Egr1 overexpression is correlated with the loss of its co-repressor NAB2 in primary prostate carcinoma. This disruption of the balance between Egr1 and NAB2 expression results in a high Egr1 transcriptional activity in prostate carcinoma cells (1). A recent study based on the cross breeding of $Egr1^{-/-}$ mice with TRAMP mice showed significantly delayed prostate tumor formation in the Egr1-deficient TRAMP mouse compared with TRAMP-$Egr1^{+/+}$ mice (2). The TRAMP mouse is a well known model of prostate cancer (20) in which tumors progress to metastases in a window from 8 to 24 weeks of age. Although Egr1 loss did not appear to prevent tumor initiation, Egr1 deficiency delayed the progression of prostate tumors in these mice. Significantly, several gene products associated with aggressive prostate cancer such as TGF-β and insulin-like growth factor II (37, 60) have been identified as regulated by Egr1. These observations strongly suggest that Egr1 is involved in prostate cancer progression despite its known role as a tumor-suppressor in several other types of human cancers (29).

In this present study on the role of Egr1, we have used the tumorigenic C2 prostate cancer cell line which was established from a prostate tumor from a single TRAMP mouse tumor. These tumorigenic cells express a high constitutive level of Egr1 protein. Transcriptional regulation by Egr1 was assessed using Affymetrix array technology. The unique step used here was to perform a microarray analysis using cells rendered deficient in Egr1 as the comparison sample for the identification of Egr1 target genes, in prostate cancer cells. The results provide new insight into the involvement of endogenous Egr1 in proliferation and survival of prostate cancer cells by the identification of several new target genes specifically controlling growth, cell cycle progression, and the apoptosis pathway.

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[1] The abbreviations used are: TGF, transforming growth factor; AS, antisense oligonucleotide; ctl, control oligonucleotide; RT, reverse transcriptase; IκBα, inhibitor κBα.

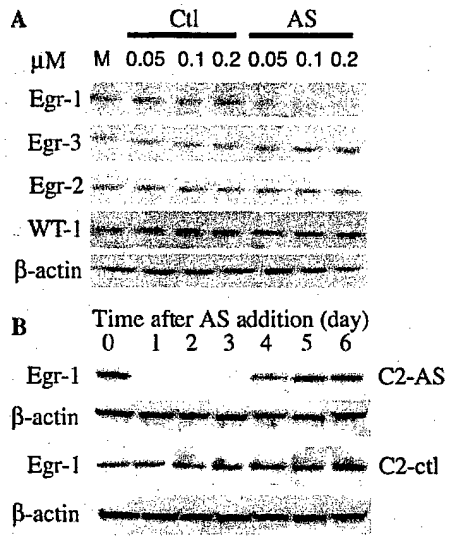


FIG. 1. **Inhibition of Egr1 expression by E5 antisense oligonucleotide.** *A*, C2 cells were transfected with the ctl, the AS, or carrier alone (*M*) for 4 h. After 24 h the cells were lysed, and samples were analyzed by Western blotting with antibodies to Egr1. Membranes were reprobed successively with antibodies to Egr-3, Egr-2, WT-1, and β-actin as internal control. *B*, proteins were extracted every day for 6 days following AS (*C2-AS*) and ctl (*C2-ctl*) transfection. Samples were analyzed by Western blotting with antibody to Egr1 and antibody to β-actin to control for protein loading.

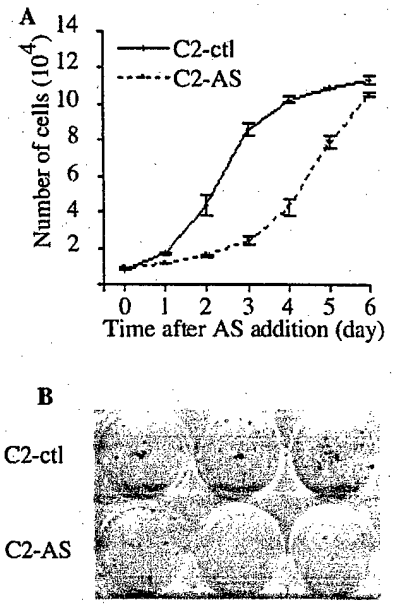


FIG. 2. **Effect of Egr1 inhibition on proliferation.** *A*, proliferation assay. C2 cells were transfected with ctl (*C2-ctl*) or AS (*C2-AS*) antisense oligonucleotide and submitted to proliferation assay for 7 days. Each day from day 0 (D0) to day 6 (D6), the number of cells of C2-ctl (*solid line*) and C2-AS (*dashed line*) was counted and plotted as the mean of three separate experiments. *B*, colony forming assay. C2 cells were transfected with 0.1 μM ctl or AS antisense oligonucleotide for 4 h. After 16 h, 200 cells were placed in each well of six-well plates in RPMI medium containing 0.1 μM antisense oligonucleotides. After 8 days, the colonies were stained with 2% crystal violet.

MATERIALS AND METHODS

Cell Culture and Transfection Condition—C2 TRAMP cells were grown as described elsewhere (20). The cells were seeded into 35-mm dishes at a density of 100,000 cells per well 1 day before transfection. The transfection was performed as described by the manufacturer with the GenePorter reagent (16 μl) (Gene Therapy Systems, Inc, San Diego, CA) and 0.1 μM antisense oligonucleotide (AS or ctl). Sequences of the AS and mismatch control oligonucleotide (ctl) were used as described (65). The sequence of ctl oligonucleotide corresponds to AS sequence with 4 bases mutated.

Proliferation Assay and Cell Death Measurement—One day before transfection the cells were seeded in duplicate into 35-mm dishes at a density of 70,000 cells per dish. At day 0 cells were transfected as described above. 4 h later the cells were harvested for counting and for protein and total mRNA extraction. This procedure was repeated each day after transfection according to a time course from day 0 to day 6.

The day after transfection, the cells were ultraviolet-C (UVC) irradiated (40 J/m^2) in a Stratalinker (Stratagene, La Jolla, CA) or treated with 100 ng/ml of Fas L recombinant protein (Oncogene Research Products, Darmstadt, Germany). One or two days after UVC irradiation or 9 and 18 h after Fas L treatment, detached and trypsinized cells were pooled and incubated with 0.2% trypan blue to determine the percentage of dead cells.

Colony Forming Assay—C2 cells were transfected as described above. After 16 h the cells were counted and seeded into 6 well plates (200 cells/well) in RPMI medium with 0.1 μM of antisense oligonucleotide. After 8 days incubation at 37 °C, the colonies were stained with 2% crystal violet.

Oligonucleotide Microarray Analysis—The protocol recommended by Affymetrix (www.affymetrix.com) was used for mRNA quality control and gene expression analysis from C2 cells transfected either with AS or ctl oligonucleotides. The probes were hybridized to Affymetrix MGU75Av2 arrays representing ~12,000 mouse transcripts. Detailed protocols for data analysis and documentation of the sensitivity, reproducibility, and other aspects of the quantitative microarray analysis using Affymetrix technology were used as reported previously (39).

Quantitative Real-time One-step RT-PCR and Western Blot—mRNA expression level was quantified by real-time one-step RT-PCR using the LightCycler-RNA-Amplification Kit SYBR Green I (Roche Molecular Biochemicals) according to the manufacturer's instructions. A standard curve from several dilutions of a sample of total RNA was established to calculate the relative amount of each gene. Values were then normalized to the relative amounts of glyceraldehyde-3-phosphate dehydrogenase determined from a similar standard curve. Each gene was amplified using the appropriate specific primers (sequences available upon request).

For the Western blot analysis, proteins were blocked and reacted with antibodies to Egr1 (C19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse and human cyclin D2 (sc-593 and sc-181, Santa Cruz Biotechnology, Inc.), p19^{ink4d} (sc-1063, Santa Cruz Biotechnology) or CD95 (anti-mouse Fas/TNFRSF6 (CD95) antibody, R & D Systems, Inc., Minneapolis, MN).

Chromatin Immunoprecipitation Assay—To cross-link protein on DNA targets the cells were incubated in 1% formaldehyde at 4 °C during 30 min. After extraction as described elsewhere (13), the chromatin was fragmented by sonication to obtain an average size of 1.5-kb DNA fragment. The DNA fragments mix was then immunoprecipitated using a specific Egr1 antibody and a non-immune serum as a negative control. After cross-link reversal as described elsewhere (13), the screening for identification of the regulatory sequence of captured Egr1 target genes was performed by PCR using the following primers located in the 5′ regulatory sequences of the following genes: p19^{ink4d}, 5′-ctggtcgctgcacgctgac-3′ (forward) and 5′-agtggataccggtggactgt-3′ (reverse) (−599 and −1, respectively, from the ATG); cyclin D2, 5′-ggcgagctgaggagagccg-3′ (forward) and 5′-ctccatagccagccggcca-3′ (reverse) (−269 and +6, respectively, from the ATG); cyclin G2, 5′-ccagcatccccaagctact-3′ (forward) and 5′-cttcatctgcagcaaatacacc-3′ (reverse) (−601 and +6, respectively, from the ATG); Mad, 5′-aagcggccggtggccgc-3′ (forward) and 5′-gctgtcgccatcctgcacc-3′ (reverse) (-48 and +11, respectively, from the ATG); CD95, 5′-cagtggtgagtcagtgggttt-3′ (forward) and 5′-gacagcccagatccacagcat-3′ (reverse) (−272 and +345, respectively, from the ATG).

Genomic DNA input was used as a control for the amplification efficiency of each primers pair. Non-immune immunoprecipitated DNA and DNA immunoprecipitated from AS-transfected C2 cells were used as negative controls. The amplified products were resolved on 2.7% agarose gel.

RESULTS AND DISCUSSION

AS Antisense Oligodeoxynucleotide Efficiently Inhibits Egr1 Expression—To examine the functional significance of Egr1 overexpression in prostate cancer cells, we inhibited its expression using an AS in TRAMP C2 prostate cancer cells. To assess the efficiency and the specificity of AS, we performed Western

TABLE I

Affymetrix analysis of genes regulated in C2 cells that express Egr1 constitutively compared with antisense treated cells

For each gene, the -fold induction (Affymetrix ratio), its function (gene function), any reported involvement in human prostate cancer (link with prostate cancer), and data on its regulation by Egr1 (known as Egr1 target gene) are given. TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Gene	Ratio	Function	Prostate CA link	Egr1 target gene
Down-regulated by Egr1				
SAA3 (serum amyloid A 3)	43.2	Inhibition of proliferation (53)	Not known	No
GBP1 (guanylate nucleotide-binding protein 1)	34.2	Inhibition of proliferation (24)	Not known	No
p19ink4d (Cdk4 and Cdk6 inhibitor)	12.3	Cell cycle arrest at G_1 (26, 43)	Not known	No
Mad (max dimerization protein)	9.4	Inhibition of cell growth (8)	Putative role-Myc is often high in prostate cancer cell (48)	No
Slfn2 (schlafen2)	9.3	Inhibition of proliferation (52)	Not known	No
IκBα	8.6	Inhibition of proliferation, angiogenesis, invasion, metastasis, sensitize to apoptosis (31, 63)	Yes (31)	No
βig-h3 (transforming growth factor β-induced)	6.4	Inhibition of growth (56)	Not known	No
Cyclin G2	5.6	Inhibition of cell cycle progression (27)	Not known	No
Apolipoprotein D	5	Associated with inhibition of proliferation (59)	Yes (59)	No
CD95 (Fas antigen)	4.9	Induction of apoptosis (9)	Yes (9)	Yes (15)
Decorin	4.7	Cell cycle arrest at G_1 (58, 70)	Not known	No
Bub1 (mitotic checkpoint protein kinase)	4.3	Required for apoptosis after mitotic check point (61)	Not known	No
RB-like 2 (retinoblastoma-like 2)	4	Tumor supressor (46)	Not known	No
PIAS1 (protein inhibitor of activated STAT protein)	2.9	Inhibition of cell proliferation (42) and induction of apoptosis (36)	Yes (23, 32)	No
Caspase 7	2.9	Induction of apoptosis (40)	Yes (41)	No
Nip3 (E1B 19K/Bcl-2-binding protein homolog)	2.8	Induction of apoptosis (10)	Not known	No
Siva (proapoptotic protein)	3.2	Induction of apoptosis (47)	Not known	No
Up-regulated by Egr1				
TIS11 (primary response gene)	16.7	Induced by TPA and growth factor (64)	Not known	No
LRG-21/Atf3 (transcription factor)	12.9	Expressed during lung cell proliferation and involved in macrophage activation (17, 35)	Not known	Correlated with increased Egr1 expression (35)
17β-Hydroxysteroid dehydrogenase type IV	9.1	Expressed in tumors (57)	Not known	No
DNAJ-like 2 (heat shock protein)	6	Putativement involved in cell cycle progression (G_2/M) (21)	Not known	No
High mobility group protein I, isoform C	5.1	Expressed during proliferation (11)	Not known	No
PS-2short	4.7	Inhibition of fas-mediated apoptosis (67)	Not known	No
$G\alpha_{12}$ protein	4.4	Stimulation of cell proliferation and transformation (68)	Not known	No
Cyclin D2	4.3	Induction of G_1 phase cell cycle progression (4, 51)	Not known	Correlated with increased Egr1 expression (22)
SIK-like protein	3.5	Induction of survival through nucleolus activity (45)	Not known	No
SPAF (spermatogenesis associated factor)	3.4	Involved in malignant conversion (38)	Not known	No
Fibroblast growth factor-inducible 15	3.4	Expressed during FGF-4 induced proliferation (25)	Not known	No
IGFBP-4 (insulin-like growth factor binding protein 4)	3.2	Abundant in prostate tumor cell lines, involved in stimulation of proliferation (16)	Yes (16)	No
TGFβ1	2.6	High level expression in prostate tumor cells (10, 11)	Yes (71, 72)	Yes (73)

blot analyses of the protein expression of Egr1 and other Egr family members, Egr2, Egr3, and wt1, 24 h after transfection of the antisense and control oligonucleotides (Fig. 1*A*). As seen in Fig. 1*A*, the antisense oligonucleotide strongly decreased Egr1 expression, while there was no effect on Egr2 and WT1 expression. Egr3 seems to be slightly increased when Egr1 was inhibited. In contrast, the ctl did not alter the protein expression pattern of the cells. These results demonstrated that a 24-h treatment with a low concentration, 0.1 μM, of the AS oligonucleotide efficiently and specifically inhibited Egr1 expression. To examine the time course of Egr1 inhibition in C2 cells, proteins were extracted each day for 6 days following AS transfection of antisense and control oligonucleotide-treated cells. Egr1 expression in the presence of AS was undetectable from day 1 to day 3, became detectable on day 4, and was fully restored on day 5 to day 6 (Fig. 1*B*, *top panel*). As expected, the use of the ctl did not change Egr1 expression level (Fig. 1*B*, *bottom panel*). These results show that AS is stable enough over 3 days to allow almost complete and specific inhibition of Egr1 expression for a prolonged period following a single treatment.

Egr1 Contributes to the Control of Proliferation—To determine the involvement of Egr1 in the proliferation rate of C2 cells, the growth of the cells in which Egr1 expression was inhibited by AS oligonucleotide (C2-AS) was compared with the control corresponding to C2 cells transfected with control oli-

TABLE II
Comparison of Affymetrix array with real-time RT-PCR ratio for mRNA levels

Changes in the expression level of several Egr1 target genes given in Table I were independently tested using quantitative RT-PCR analysis of RNA from C2-ctl and C2-AS treated cells. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as the ratio of C2-AS over C2-ctl values. All reactions were performed in triplicate from two different experiments, and the resulting S.E. values are also given. Positive and negative values mean, respectively, up-regulation and down-regulation in response to Egr1 inhibition (positive values indicate a down-regulation by Egr1).

Gene	Affymetrix ratio	Real-time PCR ratio
SAA3	43.2	72.7 ± 12.87
GBP1	34.2	5.3 ± 0.44
$p19^{ink4d}$	12.3	2.1 ± 0.22
Mad	9.4	4.6 ± 0.95
IκBα	8.6	8.2 ± 0.38
Cyclin G2	5.6	2.7 ± 0.56
CD95	4.9	4.4 ± 0.63
RB-like 2	4	2.0 ± 0.26
TIS11	−16.7	−3.3 ± 0.66
DNA-I-like 2	−6	−6.6 ± 0.50
$G\alpha_{12}$	−4.4	−7.6 ± 0.71
Cyclin D2	−4.3	−9.1 ± 1.23

gonucleotide (C2-ctl). Briefly the cells were transfected at day 0 with either AS or ctl and the proliferation rate was directly assessed every day until day 6 by cell counting (Fig. 2*A*). As seen in Fig. 2*A*, the proliferation rate of C2-AS cells was strongly reduced during the first 3 days after transfection and started to rise again on day 4. Between day 4 and 5 the slope of the proliferation curve was approximately equal to the slope of the control (C2-ctl cells), indicating that the cells recovered their expected proliferation rate (Fig. 2*A*). The proliferation time course was well correlated to the pattern of Egr1 inhibition seen in Fig. 1*B*. Indeed, as long as Egr1 expression was inhibited, the proliferation rate of C2 cells was markedly reduced and then resumed as soon as Egr1 expression recovered. In addition, comparison between C2-AS and C2-ctl cells in a colony forming assay showed 74% fewer colonies in C2-AS (average of 32 colonies (±6) for C2-ctl *versus* 8.3 colonies (±3) for C2-AS), suggesting that the tumorigenicity of the cells may decrease when Egr1 is inhibited (Fig. 2*B*). Furthermore, cell cycle analysis by fluorescence-activated cell sorter, performed at day 2 after transfection, showed fewer cells (about 11% less) in the G_1 phase of C2-ctl cells than C2-AS cells (data not shown). The sum of results strongly argue in favor of a role for Egr1 in the control of growth and cell cycle progression in prostate cancer cells.

Identification of Egr1 Target Genes by Affymetrix Microarray Hybridization—To determine the genes that are involved in Egr1-mediated transformation, comparative analyses of mRNA populations from C2 cells 1 day after transfection with AS or with ctl oligonucleotide were performed using Affymetrix microarray hybridization. Affymetrix analysis revealed a large number of genes (at least 180) involved in the control of proliferation, death, and malignant progression. Most of these had not previously been identified as part of an Egr1 signaling pathway. Although many genes are direct Egr1 target genes, others could be indirectly regulated by Egr1 or modulated after the change of the physiological behavior of the cells due to the inhibition of Egr1 expression. However it is important to consider that those genes could be as important as the direct target genes to maintain, potentiate, or regulate Egr1 effect. Genes displaying the highest Affymetrix expression changes upon treatment with AS are listed in Table I.

To confirm the Affymetrix microarray analysis results, the expression of some genes listed in Table I was independently tested by quantitative real-time RT-PCR. In these experiments, total RNA extracts from Egr1 expressing and non-expressing C2 cells were used as templates. The -fold induction/repression calculated from real-time RT-PCR assays compared with the corresponding ratio determined in the Affymetrix analysis (Table II), produced remarkable concordance. The induction or repression of specific target genes by Egr1 was in the same direction in all cases examined and commonly exhibited a similar degree of change. Indeed, the Pearson correlation coefficient of the Affymetrix and real-time PCR results was 0.78, which is significant ($p = 0.008$, χ^2). These results confirm the reliability of the Affymetrix analysis.

Examination of Table I reveals several candidate genes already identified as Egr1 targets, such as transforming growth factor beta 1 (37, 60) and CD95 (15). Expression of other genes such as transcription factor LRG-21/Atf3 and cyclin D2 is known to be correlated with an increase of Egr1 expression (22, 35). Furthermore, several genes identified here, have been directly linked to human prostate cancer. Indeed, inhibitor κBα (IκBα) was shown in several prostate cancer cell lines to inhibit growth, angiogenesis, and metastasis by inhibition of NF-κB activity (31). Mad, by interacting with Max, is known to prevent the transforming effect of Myc by inhibition of the Myc/Max association (8). In addition, Myc is often found to be overexpressed in prostate cancer cells (48). Accordingly, Egr1 could promote Myc-induced transformation by down-regulation of Mad expression. Apolipoprotein D secretion is associated with steroid-induced inhibition of cell proliferation in the LNCaP human prostate cancer cell line (59). Expression of this protein is low in prostate cancer cells and can be modulated by steroid hormones and other factors involved in the control of cell proliferation (59). Caspase 7 and CD95 (Fas/APO) are known to be involved in the apoptotic response in various prostate cancer cell lines (9, 40). Interestingly all these genes, which behave as tumor suppressors, are down-regulated by Egr1 in C2 prostate cancer cells. On the other hand, genes like IGFBP-4, which stimulates cell proliferation in ALVA31 and M12 human tumor prostate cells (16), and TGF-β1, which is strongly expressed in prostate cancer cells (71, 72), are up-regulated by Egr1.

In summary, the genes that are involved in cell cycle progression, malignant transformation, or inhibition of apoptosis are all up-regulated by Egr1, while those involved in growth inhibition and apoptosis are repressed (Table I). Hence constitutive expression of Egr1 in prostate cancer affects the balance between survival and tumor suppression.

Characterization of Egr1 Regulation—As seen in Fig. 1*B*, efficient inhibition of Egr1 expression occurred for 3 days after AS transfection. Thus, a similar time course of expression should be expected for Egr1 target genes. Therefore, mRNA expression of cyclin D2 and $G\alpha_{12}$ protein, both known to stimulate growth and cell cycle progression (4, 68), $p19^{ink4d}$ and cyclin G2, which inhibit cell cycle progression (26, 27), were measured daily from day 0 to day 6 by real-time quantitative RT-PCR. Cyclin D2 and $G\alpha_{12}$ mRNA expression was drastically inhibited from day 1 to day 3 when Egr1 was inhibited and resumed as soon as Egr1 expression was normal (days 4–6) (Fig. 3). Similarly, synthesis of cyclin G2 and $p19^{ink4d}$ mRNAs was increased until day 3 when Egr1 expression was low (Fig. 3), and normal expression was restored on day 4. These results were not observed upon treatment with the ctl oligonucleotide, demonstrating that Egr1 expression is absolutely required for full mRNA expression of cyclin D2 and $G\alpha_{12}$ and to repress $p19^{ink4d}$ and cyclin G2 mRNA synthesis. The duration of this regulation (at least 3 days) demonstrates that no other tran-

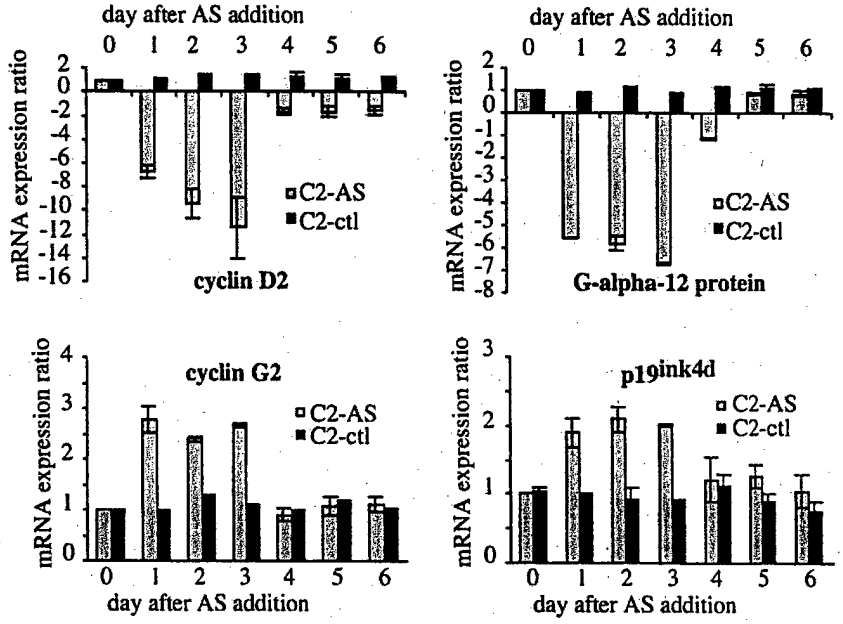
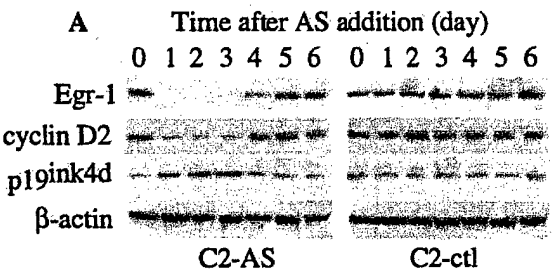


FIG. 3. **Time course of mRNA expression.** Cyclin D2, $G\alpha_{12}$ protein, cyclin G2, and $p19^{ink4d}$ mRNA expression were determined by one-step real-time RT-PCR. Expression levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase expression and the ratio between each day *versus* day 0 was calculated as -fold induction. All reactions were performed in duplicate from two different samples corresponding to C2 cells transfected with ctl (*black columns*) or AS (*gray columns*) antisense oligonucleotide.



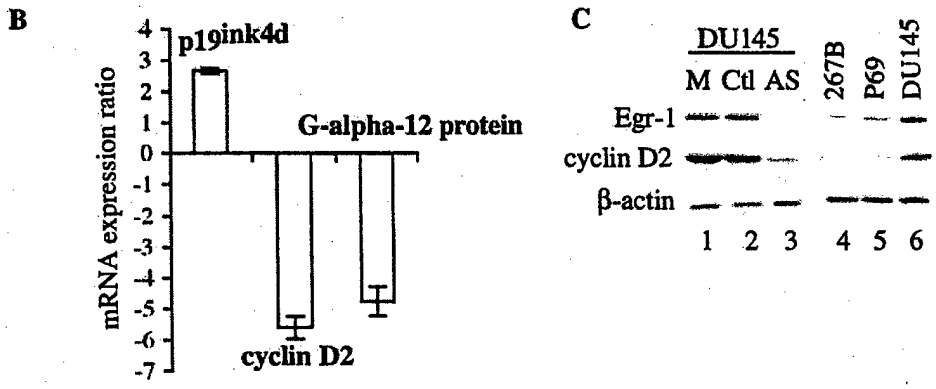


FIG. 4. **Time course of protein expression.** *A*, time course regulation of cyclin D2 and $p19^{ink4d}$ protein expression. Protein extracts from C2-ctl and C2-AS cells were analyzed as described in Fig. 1, *A* and *B*, by Western blotting with antibodies to Egr1, cyclin D2, $p19^{ink4d}$ using β-actin as a loading control. *B*, cyclin D2, $G\alpha_{12}$ protein, and $p19^{ink4d}$ mRNA expression were determined by one-step real-time RT-PCR in human prostate DU145 cells. Expression levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase expression, and the ratio between AS condition *versus* ctl oligonucleotide was calculated as -fold induction. *C*, protein extracts from DU145 (*lanes 1, 2, 3,* and *6*), 267B (*lane 4*), P69 (*lane 5*) cells were analyzed by Western blotting with antibodies to Egr1, cyclin D2, and β-actin.

scription factor compensates for the lack of Egr1 function in these cells.

To test whether mRNA regulation mediated by Egr1 is reflected at the protein level, immunoblotting analysis was performed on proteins extracted from C2-AS and C2-ctl cells from day 0 to day 6 after transfection. In these experiments cyclin D2 and $p19^{ink4d}$ protein expression level was assessed. The results showed a time-dependent repression of cyclin D2 and an increase of $p19^{ink4d}$ protein expression (days 1–3) (Fig. 4*A*) in antisense treated cells, which matched the time course of their mRNA expression patterns (Fig. 3). These results confirm that Egr1 inhibition by antisense is efficient enough to modulate Egr1 target gene expression at the protein level. In addition, the cyclin D2 and $p19^{ink4d}$ time-dependent protein expression patterns (from day 0 to day 6) are also highly correlated to the difference found in the cell cycle analysis and in the proliferation rate (Fig. 2*A*) between C2-AS and C2-ctl cells. This finding corresponds to their activities in the regulation of cell cycle progression. Thus cyclin Ds are required for cell cycle progression and overexpression of INK4 family proteins is responsible for the G_1 phase arrest (54, 55). Interestingly, cyclin D2 is found to be up-regulated by Egr1, while $p19^{ink4d}$ expres-

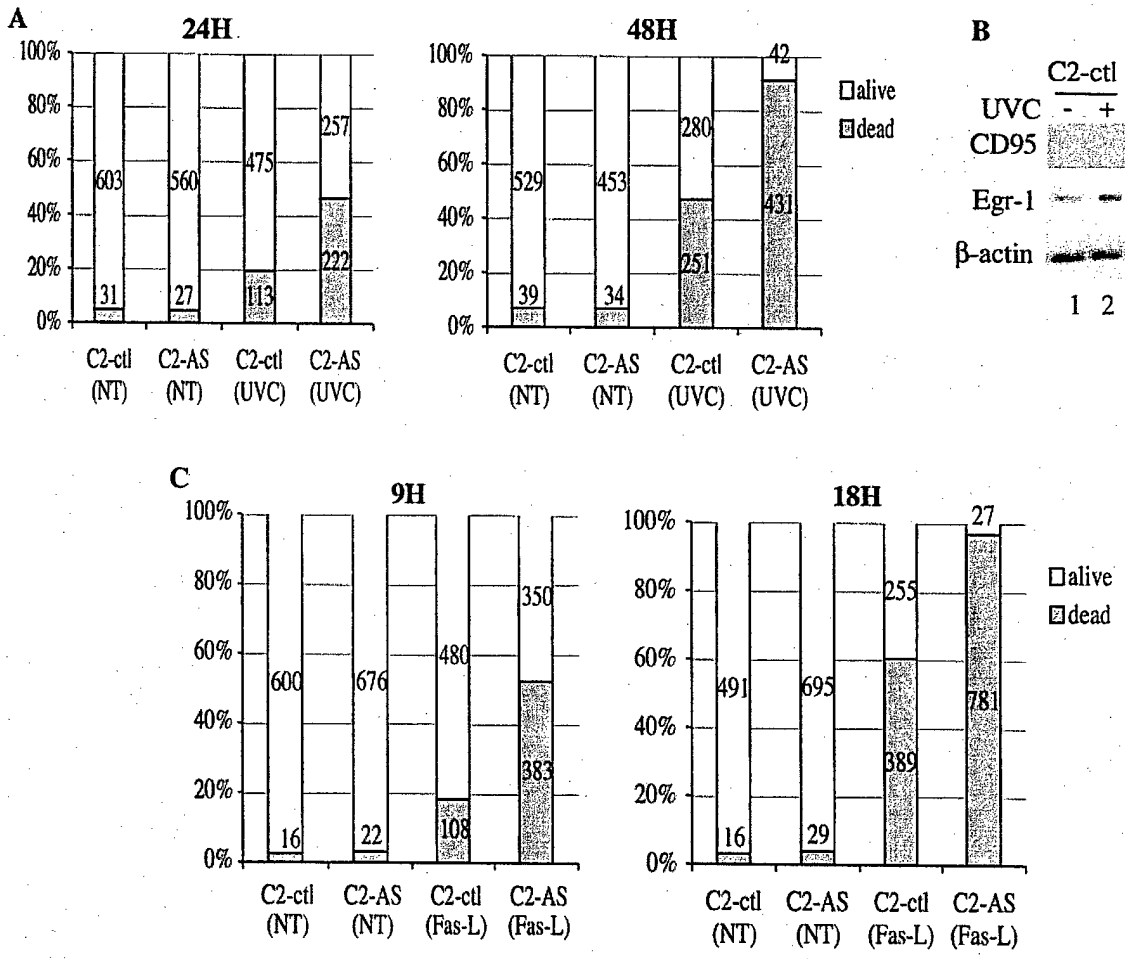


FIG. 5. **Inhibition of Egr1 expression increases sensitivity to apoptotic stimuli.** *A*, C2-ctl and C2-AS were exposed or not to UVC radiation (40 J/m^2). One and two days later dead cells were determined by trypan blue staining. The blue staining dead cell count is shown as a percentage of the total cells, and the absolute number of dead and alive cells is reported within the bar chart. *B*, C2-ctl and C2-AS were exposed (*lanes 2* and *4*) or not (*lanes 1* and *3*) to UVC radiation (40 J/m^2). Twenty-four hours later proteins were extracted and subjected to analysis by Western blotting with antibodies to Egr1 or CD95. β-Actin level were used as a loading control. *C*, Fas L-mediated apoptosis. C2-ctl and C2-AS were treated or untreated with 100 ng/ml Fas L for 9 and 18 h as described under "Materials and Methods." Dead cells were determined by trypan blue staining and reported as described above.

sion, a cyclin D2-dependent kinase inhibitor (5), is repressed. Therefore Egr1, by reciprocally regulating the levels of p19^{ink4d} and cyclin D2, would stimulate cell cycle progression and play a prosurvival role in prostate cancer cells.

To test the generality of these results, we examined p19^{ink4d}, cyclin D2, and Gα_{12} protein expression by real-time RT-PCR in the human prostate cancer cell line, DU145, transfected either with AS or ctl oligonucleotide. As in C2 cells, Egr1 expression is constitutively high in DU145 and strongly inhibited by the antisense oligonucleotide (Fig. 4*C*, *left panel*). In DU145, Egr1 regulation of these genes appeared to be the same as the regulation observed in the C2 mouse model (Fig. 4*B*). Furthermore cyclin D2 protein expression is also strongly repressed during the inhibition of Egr1 expression, indicating that Egr1 is required to maintain cyclin D2 protein expression level in DU145 as well as in mouse TRAMP C2 cells (Fig. 4*C*, *left panel*). To examine Egr1 and cyclin D2 expression during human prostate cancer progression, we tested three additional cell lines, normal 267B1 prostate epithelial cells, low tumorigenic P69 cells, and aggressively tumorigenic DU145 human prostate cells. While Egr1 expression is similar in normal human prostate 267B and P69 cell lines, it is overexpressed in DU145. Thus cyclin D2 expression correlates with Egr1 expression in these cell lines and is strongly expressed only in the aggressive tumorigenic DU145 cells (Fig. 4*C*, *right panel*). These results support the relevance of C2 cells as a model to identify new Egr1 target genes in prostate cancer.

Egr1 Desensitizes the Cells to Fas L-induced Apoptosis—Egr1 may also play a role in promoting prostate cancer by affecting prostate cell survival (30) or apoptosis (65), and this was tested next. C2-AS and C2-ctl cells were UVC-irradiated, and dead cells were counted by trypan blue staining 24 and 48 h later. While less than 20% of the C2-ctl cells were dead 24 h following irradiation, almost 50% of C2-AS cells were dead (Fig. 5*A*). Furthermore, at 48 h following irradiation, less than 50% of control cells *versus* 95% for C2-AS cells had died (Fig. 5*A*). These differences demonstrate a critical role for Egr1 in response to stress. Indeed, endogenous expression of Egr1 is not only required for full proliferation of C2 cells but also to decrease sensitivity to radiation, a widely observed phenomenon of human prostate cancer cells (28).

Affymetrix analysis (Table I) revealed several genes that are down-regulated by Egr1, such as caspase 7 (6, 40), Bcl-2-binding protein homolog Nip3 (10) and CD95 (Fas antigen) (9), a gene widely involved in apoptosis pathways. CD95, a member of tumor necrosis factor receptor family, is referred as "death receptor" because of its ability to transduce death signals. On the other hand, the gene PS-2short (up-regulated by Egr1, see Table I) is involved in inhibition of Fas-mediated apoptosis (66,

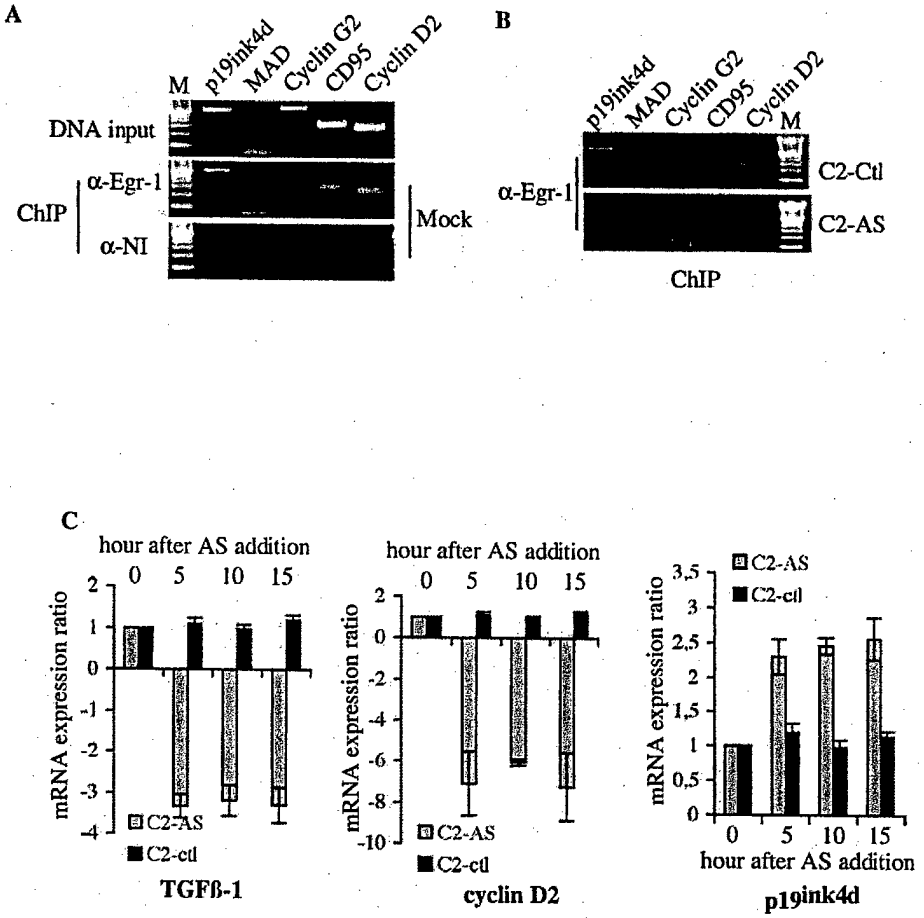


FIG. 6. **Egr1 binds directly to p19^{ink4d}, Mad, CD95, and cyclin D2 regulatory sequences.** C2 cells were transfected (*B*) or not (*A*) with AS and ctl oligonucleotides. The cells were chromatin cross-linked and then immunoprecipitated with specific Egr1 antibody or nonimmune control antibody. The detection of each gene in the captured fragment mix, was performed by PCR as described under "Materials and Methods." *A*, the *top*, *middle*, and *bottom panels* show, respectively, PCR products from the genomic DNA input, Egr1-specific immunoprecipitation samples, and the non-immune control from untransfected C2 cells (*Mock*). *B*, the *top* and *bottom panels* show, respectively, PCR products from Egr1-specific immunoprecipitation samples from C2 transfected with ctl and AS oligonucleotides. *C*, cyclin D2, p19^{ink4d}, and TGF-β1 mRNA expression were determined by one-step real-time RT-PCR. Expression levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase expression, and the ratio between 5, 10, and 15 h *versus* 0 h was calculated as -fold induction.

67), therefore supporting a role for Egr1 as anti-apoptotic agent in prostate cancer cells.

Egr1 regulation of CD95, although confirmed at the mRNA level by real-time PCR (Table II), was also tested for protein expression in C2-ctl and C2-AS cells treated or not by UVC irradiation. In C2-ctl cells, UVC treatment led to a significant increase of Egr1 expression, which was strongly inhibited by AS (Fig. 5*B*, *C2-AS*). CD95 expression appeared to be undetectable in C2-ctl-treated cells but was clearly expressed in C2-AS-treated cells (Fig. 5*B*). After UVC treatment CD95 expression was strongly increased in C2-AS, while it was only slightly expressed in C2-ctl (Fig. 5*B*). These results confirm at the protein level the efficient inhibition of CD95 expression by Egr1. This mechanism of repression is all the more relevant, since it is still effective even after a strong stress stimulus.

To assess whether this difference in basal CD95 expression could be reflected as responses to Fas L mediated apoptosis, we treated C2-ctl and C2-AS cells for 9 and 18 h with Fas L and counted the percentage of dead cells by trypan blue staining. As expected from CD95 protein expression profile (Fig. 5*B*), C2-AS were more sensitive to Fas L-mediated apoptosis. Indeed, at 9 h after treatment, 52% of cells were dead in C2-AS *versus* 18.5% in C2-ctl cell cultures (Fig. 5*C*). This difference in the resistance to cell death between C2-AS and C2-ctl cells, although lower, was still present after 18 h treatment, with 96% of dead cells compared with 60%, respectively (Fig. 5*C*). Therefore high constitutive Egr1 expression delays apoptosis of prostate cancer cells mediated by Fas L, in part by down-regulating CD95 expression. The significance of the CD95 signaling pathway in prostate apoptosis has also been demonstrated in the normal rat prostate following castration (14). In addition, further studies have demonstrated the involvement of CD95 in sensitizing prostate cancer cells to undergo apoptosis after chemotherapeutic agent or irradiation treatments (12, 33). These results illustrate well a "desensitizer role" of Egr1 in the cell death response and suggest that sensitization to Fas-mediated apoptosis by the inhibition of Egr1 expression could become an attractive therapeutic mechanism. Furthermore this experiment presents corroborating evidence that the modification of gene expression by Egr1 is a major player in the pathological responses of prostate cancer cells.

p19^{ink4d}, Mad, CD95, and Cyclin D2 Are Directly Transcriptionally Regulated by Egr1—Gene chip and real-time PCR technologies are powerful and sensitive enough to accurately evaluate the differential expression between two mRNA populations, but do not determine whether the regulation by Egr1 occurs directly or indirectly. Therefore, we performed chromatin cross-linking and immunoprecipitation assays (ChIP) to screen upstream regulatory sequences of five examples of putative Egr1 target genes indicated by the Affymetrix analysis. For this experiment untransfected, AS and ctl oligonucleotide-transfected C2 cells were used. After chromatin cross-linking in

living cells, Egr1 became covalently fixed to its DNA target. These captured target DNA fragments were then recovered by specific Egr1 immunoprecipitation and purification. Non-immune serum immunoprecipitation was used as the negative control and C2 genomic DNA was used to assess amplification efficiency of each primer pair. Primers were designed to specifically recognize 5′ regulatory sequences of $p19^{ink4d}$, Mad, CD95, cyclin G2, and cyclin D2, to detect their presence in the captured DNA fragments by polymerase chain reaction. 5′ regulatory sequence analysis of each of these genes showed several putative Egr1 and Sp-1 binding sites. $p19^{ink4d}$, Mad, CD95, and cyclin D2 yielded an amplified product from untransfected (*Mock*) (Fig. 6*A*) and ctl oligonucleotide-transfected template (Fig. 6*B*) that showed the same migration pattern as the genomic control input, while cyclin G2 was not detected (Fig. 6, *A* and *B*). Since no amplification was found for the control non-immune serum template (Fig. 6*A*) and the AS oligonucleotide-transfected template (Fig. 6*B*), these results indicate that the successfully amplified fragments were bound by Egr1 *in vivo* and therefore indicate the direct regulation of $p19^{ink4d}$, Mad, CD95, and cyclin D2 by Egr1. Furthermore, to rule out the possibility that these genes could be regulated in consequence of the inhibition of the proliferation, we performed a kinetic study of the regulation of TGF-β1, a well known Egr1 target gene (73). Since AS oligonucleotide is effective at 5 h after transfection (data not shown), we performed the kinetic analysis at 5, 10, and 15 h. As for TGF-β1, the modulation of cyclin D2 and $p19^{ink4d}$ expression occurred at 5 h after AS addition corresponding to the onset of Egr1 efficient inhibition (Fig. 6*C*). Taken together these results indicate that many of the Egr1 target genes identified in our study may be regulated directly by Egr1.

CONCLUSIONS

Our study provides new insight on the activities and mechanisms of Egr1 in prostate cancer cells. We propose that Egr1 promotes cell growth and desensitization to death by regulating a set of genes known to be very important in cell cycle progression, growth, and apoptosis. Therefore, constitutive Egr1 expression observed here in prostate cancer cells is likely to promote both tumor cell growth and progression. We suggest that our results extend the findings of Milbrandt and co-workers (2) in that they indicate the mechanistic basis of the role of Egr1 in cancer growth as well as progression. Our study confirms for the first time in prostate, the growth enhancer role of Egr1 previously observed in other cellular systems such vascular smooth muscle and rat kidney tumor cells (19, 49). However, these roles are tissue-specific, because in breast cancer, fibrosarcoma, and glioblastoma, Egr1 behaves as a tumor suppressor gene (7, 29) that can be required for maximal sensitivity to irradiation (3, 65). Further comparisons of the identity and the regulation of Egr1 target genes from these different tissues will explain this functional discrepancy.

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Review

Egr1 Signaling in Prostate Cancer

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ABSTRACT

Egr1 is a multifunctional transcription factor regulating a remarkable spectrum of cellular responses from survival to apoptosis, growth to growth arrest, differentiation to transformation, senescence as well as memory and learning effects. In prostate cancer, Egr1 levels are constitutively high and closely linked to cancer development and progression. This zinc-finger protein is a short-lived, immediate early growth response gene known to be induced by a large number of extracellular stimuli such as irradiation (all wavelengths tested), hypoxia, hyperoxia, chemotherapy agents, and more. Therefore the target genes that Egr1 regulates in prostate cancer cells play an important role in generating many of the cellular responses that characterize these cells. After Egr1 binds to its binding sites on gene promoters, specificity of response is determined by whether Egr1 transcriptionally up- or downregulates the target genes. Expression microarray analyses combined with binding data promise new ways to identify stage specific cancer markers, to aid in patient risk assessment and in therapeutic choices.

EXPRESSION OF EGR1 IN PROSTATE CANCER

We have recently reviewed the known target genes and expression of Egr1 in prostate cancer.[1] Egr1 is an immediate Early Growth Response gene induced in response to serum stimulation of quiescent or growing cells. The alternate names for Egr1 reflect the mode of its discovery. The cloning of the Egr1 gene in mouse cells and its activity in development, neuronal activity and differentiation was described by Sukhatme et al.[2] Egr1 is overexpressed in human prostate tumors[3-5] but is low in or absent from normal prostate tissue. Moreover, the level of Egr1 increases with the degree of malignancy as measured by the Gleason score[6] of the tumor.[3] This is significant and specific to prostate tumor cells because in mammary, lung and glial tumors, Egr1, is not over-expressed but is low or absent.[7-9] The Wilm's tumor gene, WT1, (at least 2 of its 4 splice variants) and Egr1 may compete for the same DNA binding motif and could impact the effect of Egr1 in tumor progression[10] especially in relation to the insulin-like growth factor gene system. Although somewhat restricted in expression pattern, WT1 is expressed in prostate cancer and may play distinct and Egr1-competitive roles there by repressing the same genes that Egr1 induces. In prostate and blood cells both WT1 and Egr1 may be coexpressed and may compete for the same gene targets. Tumorigenicity is driven in part by Insulin-related growth factor 1 and II (IGF-I and -II) in early prostate cancer, and these factors become elevated in the serum of cancer patients.[11,12] IGFs stimulate the growth and survival of prostate epithelial cells[13] and the IGF-II gene is known to be up-regulated by Egr1.[14] Other growth factor target genes of Egr1 are Platelet-derived growth factors A and B (PDGF),[15,16] EGF family members[17-21] and Fibroblast growth factors (FGF).[22,23] Over-expression of FGFs and their receptors[24,25] stimulates growth and the motility of prostate and endothelial cells. Since Egr1 expression is induced by growth factors, autostimulatory loops may synergize with the growth effect. This would lead to further growth factor production and genetic instability of proliferating prostate cells and hence to tumor progression.

An important role for TGFβ1 in prostate cancer is likely through several distinct mechanisms such as the insensitivity of TGFβ-R in prostate cells to this cytokine.[26] TGFβ1 is normally growth inhibitory to epithelial cells in vitro, however, in vivo, TGFβ is stimulatory to prostate epithelial tumor cell growth[27] and is a known target gene up-regulated by Egr1 in many cell types.[28] Some opposing effects of Egr are due to the presence or absence of NAB1 and NAB2,[29-31] natural repressors of Egr family transactivating activity. NAB2 is also inducible by the same stimuli as Egr1 and is also a factor to be considered where angiogenesis is seen.[32] However, NAB2 expression is lost in the majority of primary

prostate tumors and this results in high Egr1 levels[33] and may explain the constitutively high levels of Egr1 in some prostate cancer cell lines.

The androgen-signaling pathway plays a critical role in prostate cancer development and progression. It has been demonstrated that the WT1 tumor suppressor gene product binds to multiple sites in the androgen receptor (AR) promoter and transcriptionally represses the AR gene promoter in vitro.[34] Furthermore, Egr1 protein has been reported to bind AR in the cytoplasm of prostate cancer cells, stimulating AR transfer to the nucleus, where it binds to AR-responsive elements in the promoter of at least one prostate-specific gene (Prostate–specific Antigen (PSA), thus stimulating its expression.[35]

In summary, the checks and balances that regulate prostate growth and survival are complex. Egr1 appears to play roles that favor transformation and angiogenesis, enhanced by growth factor effects, by decreased WT1 activity, enhanced AR activity but modulated by the natural opposing gene products, NAB1 and NAB2.

EGR1 MOUSE MODELS AND PROSTATE CANCER

Mouse models using knockout and transgenic mice support the conclusion that Egr1 is required for tumor progression.[10] The importance of Egr1 to the progression of prostate cancer was demonstrated by using two transgenic mouse models of prostate cancer, CR2-Tag[36] and TRAMP[37] mice that produce prostate tumors from 10 to 23 weeks after birth, respectively. Cross-breeding of these transgenic mice with Egr1 knockout mice indicated that Egr1 deficiency significantly delayed the progression from prostatic intra-epithelial neoplasia to invasive carcinoma.[10] Abdulkadir, et al. could not find evidence that Egr1 played a role in tumor initiation rate however. The same group also demonstrated by cDNA microarray analysis that many of the growth factor genes mentioned above, are over-expressed in human prostate cancer cells over-expressing Egr1[38] (and see below).

A role for Egr1 in prostate cancer was supported by an in vivo mouse study where TRAMP mice were injected intraperitoneally every other day with antisense Egr1 oligonucleotides. The control mice were treated with scrambled or negative oligonucleotides. The antisense treated TRAMP mice still produced prostate tumors but the rate of tumor incidence at 32 weeks of age was 37% ($p = 0.026$) compared to control injected mice which was 87%.[39] This result shows that the inhibition of Egr1 expression may be a future therapeutic treatment to decrease the growth and malignancy of prostate cancer.

The prostate cells derived from TRAMP mouse prostate tumors were established in culture and proved to be very good models. Studies using antisense Egr1 to reduce the expression of Egr1 in C2 mouse prostate cells derived from the TRAMP mouse, indicated that the loss of Egr1 expression inhibits prostate cell growth.[40] This implies that Egr1 plays a role in proliferation as well as progression of prostate cancer. This is an important difference with the conclusions of Abdulkadir, et al.[10] indicating that the Abdulkadir cross of the transgenic mouse with the Egr1 null mouse model either was not refined enough to reveal changes in the initial tumor growth stages brought about by Egr1, or that immune or other responses occurred in vivo to mask these effects on growth.

Endothelial and smooth muscle cells comprise the cell types that are generated during neo-vascularization that occurs during tumor progression. Once tumor cells are more than 5 cell diameters from a source of nutrition and oxygen, hypoxia stimulates the Egr1 gene which then accelerates angiogenesis[41-43] through its set of target genes. Several groups of researchers have become interested in the roles of Egr1 during angiogenesis, atherosclerosis and other cardiovascular diseases and one of the most important key genes in these processes is the Tissue Factor gene. Tissue factor is an coagulation/angiogenic factor produced by prostate tumor, macrophages and endothelial cells. Notably, both Tissue factor and VEGF (the endothelial cell growth factor) genes are induced by Egr1[44-46] to stimulate angiogenesis. In vivo, prostate tumor tissue growth, progression, angiogenesis are increased by TGFβ1[47] and TGFβ1 has been shown to be increased in expression in prostate tumors.[48] Since TGFβ1 is known to be transcriptionally induced by Egr1,[49] it seems logical to suggest that Egr1 is a major inducer of this cytokine in prostate cancer cells and that it affects endothelial and stromal cells in addition.

MOLECULAR PROFIING OF PROSTATE TUMORS USING EXPRESSION MICROARRAY TECHNOLOGY

The analysis of the mRNA transcription products of tumor tissues has been a promising source of putative new gene markers of specific stages of tumor progression to aid in diagnosis, risk assessment and possible new therapeutic targets. Hybridization of probes made from mRNA to DNA microarrays representing nearly all known human and over 12,000 mouse genes is the method of choice for expression analysis. Numerous precautions are necessary and have been discussed.[50] The technology has evolved in several stages but is now commercially dominated by glass microchips arrayed with oligonucleotides representing nearly 100,000 human gene variants to allow the identification of the genes that are up- or downregulated. The usual format involves the analysis of two contrasting states, such as unstimulated versus induced cells, or normal versus tumor tissues. Affymetrix holds the major share of the large market that has developed, and this is being somewhat eroded by smaller but more economical procedures using larger oligonucleotides such as 50-, 60- or 70-mers representing the coding sequences of a large portion of the human genome. Both systems use fluorescently labeled cDNA "targets" to interrogate the array of DNA "probes" spotted in known locations on the array to reveal the identity of each and every expressed gene in a semi-quantitative signal comparison of the pair of samples analyzed. The data are computed as normalized signal fold-change ratio over the control and then subjected to increasingly sophisticated data reduction and bioinformatics software programs to present the statistically relevant data in a meaningful way to clinicians and researchers.

The loss of sensitivity and specificity due to background noise in chip technology remains a significant problem in microarray analyses and all such analyses are in urgent need of better analytical programs to retrieve the real signals from the considerable amount of background that reduces the precision of the method. Nevertheless it is the high-through-put procedures that are helping to integrate huge amounts of data to reveal at least the most significant genes that are altered in expression in each condition tested. Analyses of cell lines have gradually given way to tissue analyses, especially to tumor tissues of all kinds. One drawback of expression profiling is that genes that are mutated may not be distinguished from the normal, and they may be detected at increased levels that represent the inactive product. For example, TP53 which encodes the tumor suppressor p53 protein is often detected as an expressed mRNA for this reason even when the protein product is known to be transcriptionally inactive. To confirm the results of such studies, it is necessary to validate all

important findings with quantitative RT-PCR and immunoblotting to measure both mRNA and protein levels. In addition, in the case of p53, antibodies that distinguish between the wildtype and the most common p53 mutant proteins must be used. Another problem in expression arrays that is avoided in the promoter array (see below) is that the mRNA in the tissue undergoes changes due to ischemia as the tissue is processed for routine pathological analysis or during tissue processing. For example an analysis of mRNA losses or gains during warm ischemia detected several transcription factors (Egr1, Jun B, Jun D and ATF3 at high levels at the 1 h time point but not at the zero time point, raising the possibility that genes previously associated with prostate cancer may not be relevant to tumor-expressed genes but to stress responses.[51] Therefore extra care and speed is required in collection and processing of tissues for studies using expression analyses. In addition, the more short-lived proteins including all the immediate early transcription factors will be degraded in time (an average of 90 min half-life) and validation by immunoblotting will not present good evidence either.

Two studies using expression microarrays have focused on a role for Egr1 in prostate cancer and used cell lines where extraneous gene responses were unlikely. In one study a human prostate cancer cell line, LAPC4, was infected with an adenovirus expressing Egr1 to determine the regulated genes compared with control-infected cells. Several known Egr1-regulated genes were identified as over-expressed, such as insulin-like growth factor-II IGF2), platelet-derived growth factor-A (PDGF-A), and transforming growth factor-beta1 (TGFβ1), which have previously been implicated in enhancing tumor progression.[24,52,53] Validation by QRT-PCR for several genes, including IGF2, neuron-specific Enolase, Rad, ID4 and EF-1α were shown to be well correlated with Egr1 expression. In addition, several neuroendocrine-associated genes were expressed at elevated levels.[38] The other study was mentioned above where Affymetrix chips were used to identify the genes that were regulated in mouse prostate C2 cells treated with antisense Egr1.[40] A third microarray study investigated the role of Egr1 in endothelial cells using adenoviral transfer to over-express Egr1, with the conclusion that Egr1 is a key mediator of inflammation and apoptosis in vasculature.[54]

Prostate cancers are among the most morphologically heterogeneous tumors and present special problems related to this. Dhanasekaran, et al. applied expression profiling to the study of prostate tumors at two major stages to determine the differential expression of genes. At the metastatic stage tumors are close to incurable and therefore stage markers are extremely important. The team applied expression arrays to show that of the 55 genes upregulated, the Polycomb gene EZH2, was one of the strongest markers of metastatic stages in comparison with benign tissues and these corresponded with poor clinical outcome.[55] Inhibition of EZH2 expression with RNAi resulted in significant impairment of prostate cancer cell growth, and this correlated with the effect of EZH2 group of genes as regulators of chromatin structure and hence on large-scale regulation of gene expression. Supporting evidence has emerged from a tissue microarray study[50] that in addition showed that a ratio of EZH2 to E-Cadherin is significantly correlated to recurrence of prostate cancer after radical prostatectomy. In addition, this study found that hepsin was expressed at elevated levels and hepsin was also confirmed as a progression marker in multiple other studies.

Hepsin was found to be upregulated in prostate cancers by at least 5 groups, including the Egr1 overexpression study.[38] This transmembrane serine protease is significantly correlated with malignancy in prostate cancer[56-59] and has been shown to be released into seminal fluid.[60] Hepsin was shown to be upregulated in other tumors also.[61] Interestingly, the hepsin protein product itself was shown to be growth inhibitory to at least 3 prostate cancer cell lines that were transfected to over-express hepsin[62] while hepsin was found to stimulate endothelial cell growth and angiogenesis.[63,64] Other growth- stimulating markers that are upregulated are PDGF-Rα and members of the MAPK signal pathway, reviewed by Ciro, et al.[65] several of which are known or suspected Egr1 target genes. In some prostate cancer cases the activation of the coagulation cascade was noted and Tissue Factor, a major player is worthy of mention because TF is also transcriptionally induced by Egr1 in many circumstances of stress, cardiovascular disease and angiogenesis.[66]

As mentioned above, a significant problem in prostate cancer profiling is the heterogeneous nature of the tissue, with mixtures of normal and benign epithelial hyperplasia, frank tumor tissue, stromal and endothelial cells mixed with vascular elements. Molecular profiling of this type of tissue is possible but difficult with the application of the laser capture microdissection procedure that allows the individual tissues of interest to be selected, cut out and collected for RNA extraction as separate cell types within the cancer tissue sample. Using this procedure, caveolin-1 was shown to be a tumor suppressor gene that can be detected in tumor tissue as a gene whose promoter was hypermethylated compared with normal epithelium in 90% of cases. Despite this, caveolin-1 was expressed and was detected in tumor cells and in normal epithelium in paraffin-embedded material in a manner that suggested that methylation of this gene is predictive of tumor recurrence.[67] Caveolin-1 is an autocrine/paracrine growth factor associated with androgen insensitive prostate cancer and may have a potential as a serum biomarker due to the development of an immunoassay for serum caveolin-1[68] since expression serum levels appear to differentiate between prostate cancer and benign prostatic hyperplasia.

The heterogeneity of prostate cancer was treated in a different way by Stuart, et al. in that mixed prostate cancer tissues were analyzed using Affymetrix arrays™, and in parallel, a bioinformatics and statistical method was applied to solve the problem of which cell types were responsible for which molecular profiles. This was nicely done by a team of four experienced pathologists who made independent judgments of the proportion of cellular components (BPH, stroma and tumor) of representative histological stained sections of each tumor, by well accepted pathological and histological criteria. The scores were averaged and factored into the statistical analyses of 88 prostate specimens, to arrive at a list of genes that were associated with each type of tissue within the tumor sample.[69] These results were validated by immunohistochemistry and RT-PCR to confirm that this type of in silico analysis is an important way to distinguish between tissue mixtures that would previously have defied cell specific analysis. The results indicated that stromal cells are responsible for the expression of a large proportion of the TGF-β-related genes, including the cytokine, receptors and TGFβ-inducible early growth response genes. Stromal cells and to a lesser degree tumor, expressed higher levels of FGF (2, 4, 7, 8, and 18) as well as FGF-R2 and IGF-I. Tumor cells were associated more distinctly with hepsin, RAN (a Ras member) LIM protein and CD24 among other genes expressed in tumor cells. Tumor cells were also responsible for the production of PSA (kallikrein 3) and HGF, which have been detected frequently in these array methods. New patterns of gene expression for these three lineages were produced that are relevant to novel

pathogenetic, diagnostic and therapeutic considerations. The avoidance of labor-intensive laser dissection is a clear advance for molecular profiling of each individual tumor for risk assessment and diagnosis.

DEVELOPMENT OF PROMOTER MICROARRAYS—TARGET GENES REGULATED BY EGR1

The novelty of the promoter array and its utility in rapid analysis of cancer tissue of individual patients was recognized sometime before 1998. We and others worked out the conditions that utilize antibodies to a specific DNA-binding protein (in our case Egr1 transcription factor) using chromatin immunoprecipitation (ChIP) to capture actively-regulated genomic DNA in cells[28] and to clone new genes using several different methods.[70] Recently three examples of promoter arrays used to analyze the transcription factors active in yeast have been published by the Young group and others.[71-73] The difficulties of mRNA decay during collection and preparation for expression microarray analyses are avoided by the application of a different type of analysis: to determine the target genes of specific transcription factors at a moment when the tumor tissue or tumor cell lines are alive and untreated, or after stimulation with γ-radiation or chemotherapy and responding to the insult. Several groups of researchers at different Cancer Centers have collaborated to investigate the best protocols to apply to cells and tissues, to analyze the target genes of specific transcription factors in a high-throughput fashion using promoter microarray chips. We have started with the target genes of Egr1 because this factor is rapidly induced by nearly all stimuli applied to living cells. We have found that the binding of Egr1 to its target genes differs for each stimulus and for its intensity, and this in turn affects the expressed gene population and the downstream cellular response. Radiation causes live cells to pass into a period of growth arrest, DNA repair and sometimes into apoptosis, depending on the intensity of the stimulus. In brief, the procedure allows the determination of the identity of all the target genes of Egr1 by capturing the promoters of all the regulated genes using chromatin immunoprecipitation (ChIP). The DNA is collected as a mixture of fragments on average 1kb in length, purified and labelled with fluorescent dyes Cy3 or Cy5. Control input genomic DNA is labeled with the other dye for competitive hybridization to promoter DNA on the microarray. The fluorescent signals are digitized by scanning confocal fluorimeters and analyzed by data reduction packages such as ScanArray and Quantarray, in a similar fashion to Affymetrix analyses. The main difference is that here the two fluorescent signals are produced from the same chip and are analyzed in separate channels and normalized as signal intensity over background negative controls (using a series of unrelated DNA spots from plant and bacterial sources). Then channel 1 sample signals versus channel 2 (control DNA) are calculated as a ratio and then subjected to various analytical programs to determine the significant signals from the identified spots on the array. The DNA on the array currently contains about 3300 gene promoters, specifically selected for their relevance to cancer, stress responses, growth regulation and apoptosis: in addition all the known and putative target genes of Egr1, AP-1 and Androgen receptor were selected for application of the promoter sequences to the array. Each promoter sequence is about 1.2 kb and was selected batch-style to include 1 kb of promoter and +500 downstream of the transcriptional start site, before repeat masking and primer selection from free programs on the internet. Primers were synthesized by Illumina Corp. and were used in batch PCR reactions to prepare the DNA for spotting on the chip. A similar program has been developed independently by Ren, et al.[74,75] and used to discover the mechanism for the regulation of the cell cycle and DNA repair by E2F. A similar chromatin immunoprecipitation procedure followed by target gene identification has been applied by the Farnham group using a CpG island microarray that performs a similar role to a promoter array, since CpG-rich regions make up a portion of about 60% of promoters. This group determined the role of Rb in the S phase of the cell cycle.[76,77]

Our applications of the ChIP on a chip technique are two-fold. One study uses a set of prostate cell lines that represent the stages of prostate cancer progression and is currently ongoing but involve determination of Egr1 target genes in untreated prostate cells and in cells exposed to serum stimulation, UV-C , or ionizing irradiation, or etoposide treatments, all of which induce the rapid expression of Egr1 and to different degrees in the different cell lines. Each treatment is analyzed for target gene preference in binding. The analysis shows a trend in gene targets with different malignancy levels of the 4 cell lines. The results have been verified by QRT-PCR and conventional ChIP and are under further analysis. Results so far indicate that genes that regulate the cell cycle, growth arrest, DNA repair, salvage pathways, ribonucleotide metabolism, and apoptosis dominate the lists that are emerging. This is consistent with the effects of the stimuli given to the cells that elicit immediate early transcription factors. The cells are subjected to formaldehyde cross-linking at the time when Egr1 transcription factor expression is maximally expressed. Using the same conditions and drug additions, we also collected cells for mRNA extraction and subjected the labeled cDNA to hybridization to cDNA oligonucleotide expression microanalyses to determine which genes are up- or downregulated by the stimulus to the cells. Integration of the results of the two experiments requires the application of new algorithms, new types of cluster analyses and statistical methods to extract data with acceptable confidence limits (95%). When these results are further integrated with gene function data bases, it is possible to discern gene networks and pathways, the so-called regulatory networks that have become a popular theme recently.

PERSPECTIVE

There is no doubt that high-through-put methods are required for analyses of the roles of genes in human diseases. Microarray technologies produce huge quantities of data that have to be reduced to usable interpretations of the existence, regulation and functions of gene pathways in live responsive cells. We suggest that the activities of transcription factors should be an integral part of the analyses by providing promoter binding data. New database systems that process microarray information are becoming available daily and analytical algorithms that can integrate the data with activity, binding and functional information, together with programs that produce statistically valid data analyses resulting in biological predictors are appearing. These require further refinement, together with programs that can discern false positive and false negative signals and that can distinguish background noise from low but real signals and hence will increase the sensitivity and specificity of microarray studies. When this occurs in the near future, the findings will be greatly more valuable. Today, we can suggest a handful of dominant gene products that can act as the more coarse indicators. Tomorrow, array technology development promises to bring a great deal of information in an easy-to-interpret form that will be useful in the clinics for diagnosis of disease stage and predictors of treatment options.

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Early growth response 1 protein, an upstream gatekeeper of the p53 tumor suppressor, controls replicative senescence

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The proliferation of most primary cells in culture is limited by replicative senescence and crisis, p53-dependent events. However, the regulation of p53 itself has not been defined. We find that deletion of the early growth response 1 (EGR1) transcription factor leads to a striking phenotype, including complete bypass of senescence and apparent immortal growth consistent with loss of a suppressor gene. EGR1-null mouse embryo fibroblasts (MEFs) exhibit decreased expression of p53, $p21^{Cip1/Waf1}$, and other p53 "marker" proteins. Precrisis WT but not EGR1-null cells exhibit irradiation-induced arrest. WT MEFs that emerge from crisis exhibit a mutated p53 (sequence confirmed), colony formation, and tumorigenicity. In contrast, high-passage EGR1-null MEFs retain the WT p53 sequence but with much reduced expression, remain untransformed, and grow continuously. An EGR1-expressing retrovirus restores p53 expression and sencescence to EGR1-null but not p53-null MEFs or postcrisis WT cells. Taken together, the results establish EGR1 as a major regulator of cell senescence and previously undescribed upstream "gatekeeper" of the p53 tumor suppressor pathway.

early growth response 1 gene | cancer | retrovirus | mouse embryo fibroblasts

The proliferative capacity of most primary cells in culture is limited by the induction of senescence. The senescent state depends on a number of pathways that together result in permanent cell-cycle blockade (1). In most rodent cells, induction of the tumor suppressor genes p53 and $p19^{ARF}$ are critical to the induction of senescence, as inactivation of either gene allows "bypass" of replicative senescence, leading to continuous growth (2, 3). The function of p53 is determined in part by $p19^{ARF}$ and by murine double minute-2 protein (MDM2). The study of senescence in cultured cells and its related aspects of lifespan extension and immortalization has become an experimental system of great value for understanding tumorigenesis (4). Senescent populations undergo "crisis" and deteriorate, however, rare primary mouse embryo fibroblasts (MEFs) that acquire "escape" the senescent state and mitotically expand. Most such postcrisis cells are hypotetraploid and contain mutant p53 alleles, whereas others are mutated at $p19^{ARF}$ and remain pseudodiploid (5). The potential importance of MDM2 in senescence is illustrated by amplification of MDM2 in lymphomas (6). Thus, the p53-MDM2-$p19^{ARF}$ pathway is critical for the induction of senescence. However, the upstream regulatory mechanisms controlling this pathway remain unclear.

Another factor that has been discussed as a tumor suppressor is the early growth response 1 (EGR1) transcription factor (7). EGR1 is a member of the immediate early gene family and regulates transcription of target genes through GC-rich elements. EGR1 is involved in the regulation of growth and differentiation (7). However, many human tumor cell lines express little or no EGR1 in contrast to their normal counterparts (8–10). Furthermore, EGR1 has been found to be decreased or undetectable in small cell lung and human breast tumors (11, 12) as well as in human gliomas (13). Taken together, these data suggest a potential role for EGR1 in tumor suppression.

The mechanism of growth suppression by EGR1 as well as EGR1-dependent pathways are incompletely understood. We show here that EGR1 deficiency leads to a complete bypass of replicative senescence and an apparent immortal growth of MEFs. This effect of EGR1 is found to depend on its ability to act as an upstream regulator of the p53 tumor suppressor pathway. Our results thereby establish EGR1 as a previously undescribed gatekeeper of p53-dependent growth regulatory mechanisms in replicative senescence and cell growth.

Materials and Methods

Cells, Cell Culture, and Irradiation Treatment. MEFs were prepared as described (14) from 15- or 19-day-old embryos from EGR1 WT, EGR1-null, and EGR1 heterozygous (HTZ) mice kindly provided by J. Milbrandt, Washington University, St. Louis (15). The predicted genotype and expression properties of the MEFs derived from EGR1-null and HTZ mice were confirmed by PCR-based analysis of DNA and RNA and by Western analysis of protein expression. Genotyping of MEFs derived from mice generated by Charnay and coworkers (16) was performed as described.

The p53-null MEFs were a gift from P. Puri (The Salk Institute, San Diego) and originally derived by I. Hunton in the laboratory of J. Y. J. Wang (University of California, San Diego).

For growth (proliferation) curve determinations, cells were seeded into six-well tissue culture plates at 20,000 cells per well in DMEM (high glucose) supplemented with 10% FBS and 75 μg/ml hygromycin B. Cell numbers were determined on days 2, 4, 5, and 6 by using a Multisizer II Coulter counter equipped with a channel analyzer for exclusion of noncell counts.

For irradiation experiments (Fig. 4), freshly prepared WT MEFs were isolated and compared to EGR1-null cells. All cells were seeded into six-well tissue culture plates at 70,000 cells per well grown as above and irradiated with 7.5 Gy by using a Cs source. Cell numbers were determined on days 1, 3, and 5, after irradiation or 3 days after reseeding by direct cell counting (Coulter).

Colony Formation Assay. Cells were seeded into 6-cm diameter tissue culture dishes at 600, 900, or 1,200 cells per dish and grown as above. After 10 days of culture, the colonies were stained with 2% crystal violet, and cell numbers were determined in a parallel experiment.

Tumorigenicity Assay. Six-week-old female athymic mice (Harlan–Sprague–Dawley) were placed in a pathogen-free environment.

Abbreviations: EGR1, early growth response 1; HTZ, heterozygous; MEF, mouse embryo fibroblast; Q-PCR, quantitative-real-time PCR; MDM2, murine double minute-2 protein.

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At the time of assay, postcrisis WT MEFs at passage 56 or EGR1-null MEFs at passage 62 were trypsinized, counted, washed twice in PBS, and resuspended in PBS 0.1% BSA at 7×10^6 cells/0.1 ml. The same volume of matrigel (Becton Dickinson) was added, and 7×10^6 cells (200 μl) were injected s.c. into each mouse on the dorsal-lateral surface. Mice were monitored for 16 wk for tumor formation.

Retroviral Vector Construction. A retroviral vector, pLHC-EGR1, was prepared and used as described (10). The titer was monitored by Western analysis of producer cells and supernatants. Forty-eight hours after retrovirus infection, cells were cultured in medium containing 75 μg/ml hygromycin B. After 2 wk of selection, the hygromycin-resistant colonies were used for Western analysis to assess the expression of EGR1.

Quantitative Real-Time PCR (Q-PCR) and Western Analysis. RNA expression levels were quantified by Q-PCR (Applied Biosystems 7900). Total RNA (0.5 μg) was reverse-transcribed into cDNA by using Superscript II RNase H$^-$ Reverse Transcriptase kit from Invitrogen. Q-PCR primer sequences were selected for each cDNA with the aid of PRIMER EXPRESS software (Applied Biosystems) and are available on request. Q-PCR and quantitative measurements were performed with the SYBR-Green PCR-Master Mix (Applied Biosystems) (Applied Biosystems 7700 user bulletin no. 2). The results were normalized to the relative amounts of GAPDH. For Western analysis, cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitors as described (10), and the membranes were labeled with Abs specific for EGR1 (sc-189, Santa Cruz Biotechnology); p53 (Pab246; sc-100, Santa Cruz Biotechnology); $p21^{Cip1/Waf1}$ (sc-397, Santa Cruz Biotechnology); EGR2 (sc-190, Santa Cruz Biotechnology); or actin (Sigma).

Immunoprecipitation. Cells were lysed in 150 mM NaCl/50 mM Tris (pH 8.0)/5 mM sodium EDTA/0.5% Nonidet P-40 supplemented with protease inhibitors mixture and 2 μM lactacystin β-lactone on ice. Protein (400 μg) was precipitated overnight at 4°C by using either a monoclonal mouse-specific and conformation-dependent Ab (Pab 246; sc-100, Santa Cruz Biotechnology) that recognizes WT but not mutant p53 (17) or with a mAb (ab26; Pab 240, Abcam, Cambridge, U.K.) that recognizes many mutant p53s but not WT p53 protein in its native form (18). Precipitates were solubilized in denaturing sample buffer, electrophoretically separated, and transferred to Immobilon P membranes for detection with a polyclonal p53 Ab (sc-6243).

Sequencing. RNA from pre- and postcrisis WT MEFs and high-passage EGR1-null MEFs was reverse transcribed into cDNA. PCR was performed by using the following primers for p53 (GenBank accession no. K01700): forward position, 420–438, 5′-ggcccctgtcatcttttgt-3′; reverse position, 1,164–1,183, 5′-attcagctcccggaacatct-3′. Sequence reactions of these PCR products were done by BATJ (San Diego).

Results

EGR1-Null MEFs Show Enhanced Cell Growth and Bypass Senescence. The cell growth of MEFs derived from WT mice, EGR1 HTZ mice, and EGR1-null mice was monitored by cell number counting. In EGR1-null mice, expression of EGR1 is interrupted by the insertion of a neomycin resistance gene cassette upstream of the DNA-binding domain, which introduces in-frame stop codons (15). MEFs derived from the EGR1-null mouse strain established by Milbrandt and coworkers (15) exhibit high aberrant transcript levels corresponding to the altered EGR1 locus; however, no immunoreactive protein product is expressed (Fig. 3*D*), consistent with findings from Milbrandt and coworkers (15, 19). Normally, MEFs stop dividing and go into crisis after a characteristic number of passages as illustrated in Fig. 1. This so-called "replicative senescence" is p53-dependent (5).

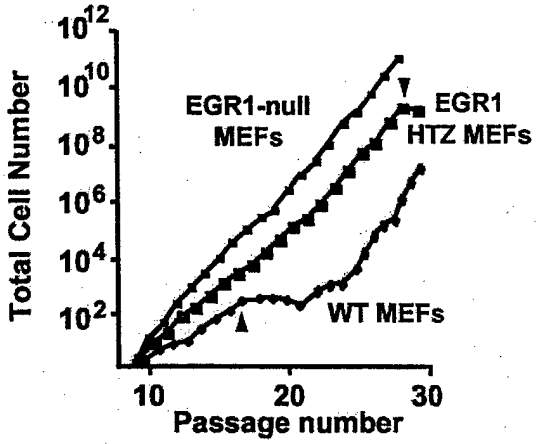

Fig. 1. EGR1-null MEFs bypass replicative senescence. MEFs from WT, EGR1 HTZ, or EGR1-null mice generated by Milbrandt and coworkers (15) were passaged every 3 or 4 days from passage 1 by counting the total number of cells and reseeding 3×10^5 cells per 60-mm dish. Accumulative cell numbers were calculated at each passage. Each growth curve is the average of two independent primary MEF isolates of the indicated genotype. Start of crisis is indicated by arrows; note that postcrisis cells are "WT MEFs" in origin only and exhibit non-WT sequences and phenotype (see text).

In our experiment, passaged WT MEFs initially underwent one population doubling in 3 days to become confluent, at which time they were harvested and reseeded. However, their growth virtually ceased by passage 17–20 (Fig. 1). After this senescent state, postcrisis "survivors" became established as permanent cell lines. In contrast, EGR1-null MEFs grew linearly for >60 passages appearing to bypass senescence. MEFs from EGR1 HTZ mice showed an intermediate growth rate, i.e., paused after 28 passages and resumed rapid growth (Fig. 1). The growth curves in Fig. 1 are the average of two independent primary MEF for each genotype. MEFs prepared from earlier embryos exhibited an essentially identical phenotype (not shown). In addition to the EGR1-null mice generated by Milbrandt and coworkers (15), we prepared and examined the growth of primary MEFs from EGR1-null mice developed by using a different plan by Charney and coworkers (16). In the MEFs derived from these EGR1-null mice, EGR1 RNA and protein levels were undetectable and they showed the same characteristics as the MEFs from Milbrandt and coworkers (not shown). In all, five complete sets of independent isolates were examined and varied only in the passage number of onset of the growth plateau for WT MEF and the prominence of the transiently reduced growth for HTZ MEFs.

These results, therefore, suggest the possibility that EGR1 is required in a gene-dose-dependent manner for the senescence response of WT MEFs observed in culture.

Expression Analysis by Using Q-PCR Reveals Decreased Expression of Several Regulators of Growth and Differentiation Such as p53, $p21^{Cip1/Waf1}$ as Well as a Number of p53 Marker Proteins in EGR1-Null MEFs. To identify EGR1-regulated genes, which may be responsible for the absence of a senescence state in EGR1-null MEFs, expression analyses were performed by using the mouse Affymetrix Gene Chip (unpublished data). Among the differentially expressed genes were the genes involved in growth differentiation and cell-cycle control. In this regard, transforming growth factor type β1 and p53 mRNA expression was decreased as well as the mRNA expression of a number of known p53 target genes, e.g., *p21*$^{Cip1/Waf1}$ (20), *GADD45* (21), *Bax* (22), and *Fas* (23) (unpublished data). We confirmed the

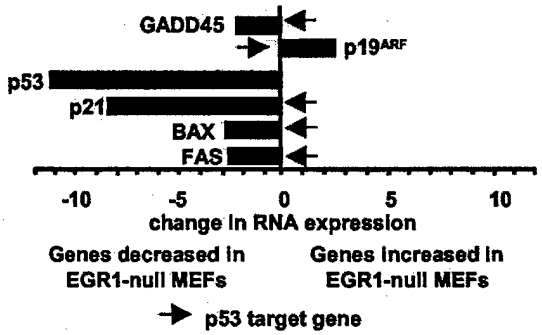

Fig. 2. Q-PCR reveals expression of genes differentially expressed between WT and EGR1-null MEFs. RNA expression levels were quantified by Q-PCR (ABI 7900). Total RNA (0.5 μg) from WT MEFs or EGR1-null MEFs was reverse-transcribed into cDNA and amplified by using the SYBR-Green PCR-Master Mix and specific primers for each cDNA as described (*Materials and Methods*). The relative amounts of each gene amplification products were calculated by reference to standard curves and were then normalized to the relative amounts of GAPDH as detected in the same run. The fold change in RNA expression from EGR1-null MEFs as compared to WT MEFs is shown. Known p53 target genes are indicated by arrows.

decreased expression of these genes by Q-PCR (Fig. 2). Target gene expression results were supported by using independent RNA preparations from WT MEFs and EGR1-null MEFs and by using different primers and expression analysis methods (e.g., semiquantiative PCR), which included confirmation of the predicted product size by visualization on agarose gels. The sum of results indicate that deletion of EGR1 is associated with decreased expression of a number of established p53-regulated genes.

Reexpression of EGR1 in EGR1-Null MEFs by Retroviral Infection Restores Replicative Senescence. To verify the EGR1 dependence of the senescence phenotype in murine MEFs, we performed reconstitution experiments by using an EGR1-expressing retrovirus system. When infected with an EGR1-expressing retrovirus, the cells became hygromycin-B resistant and showed increased steady-state protein levels of EGR1 (Fig. 3 *C* and *D*). Infection of EGR1-null MEFs with the EGR1-expressing retrovirus completely rescued the WT MEF phenotype (Fig. 3*A*). EGR1-infected cells were no longer able to bypass senescence and stopped growing 5 days after infection. In contrast, EGR1-null MEFs infected with an "empty vector" control virus became hygromycin-B resistant but did not stop growing and did not senesce (Fig. 3*A*). Similarly, WT MEFs infected with an EGR1-expressing retrovirus were not retarded in growth compared to MEFs infected with an empty vector control virus (not shown). Q-PCR demonstrated that a number of genes poorly expressed in EGR1-null MEFs became up-regulated after EGR1 virus infection (Fig. 3*C*). Among these genes is *p53* as well as known marker genes of p53 transcriptional activity such as *p21*$^{Cip1/Waf1}$ (20), *Reprimo* (24), *GADD45* (21), *MDM2* (25), *Bax* (22), and *Fas* (23). EGR1 itself was up-regulated twofold. In contrast, p19^{ARF}, known to be negatively regulated by p53 (26), was down-regulated in these cells. The regulation of a variety of p53 target genes by EGR1 reexpression indicated that p53 might play an important role in the EGR1-mediated growth suppression.

To confirm these results, we also studied the effect of retroviral-mediated expression of EGR1 on p53 and p21$^{Cip1/Waf1}$ protein levels (Fig. 3*D*). Western analysis demonstrated that p53 and p21$^{Cip1/Waf1}$ protein expression was down-regulated in EGR1-null MEFs whereas the expression of both proteins was up-regulated in EGR1-null MEFs infected with an EGR1-expressing retrovirus (Fig. 3*D*). As expected, EGR1 was not detectable in EGR1-null MEFs but was restored in these cells after EGR1 retrovirus infection (Fig. 3*D*). No changes could be observed in the expression of EGR2. Overall, these results, confirm the observations that reexpression of EGR1 in EGR1-null MEFs leads to increased expression of p53 and p21$^{Cip1/Waf1}$ at the RNA and protein levels.

Reexpression of EGR1 Does Not Restore Replicative Senescence in p53-Null MEFs. Our experiments suggest that EGR1 might regulate the induction of the senescent state through the *p53* tumor

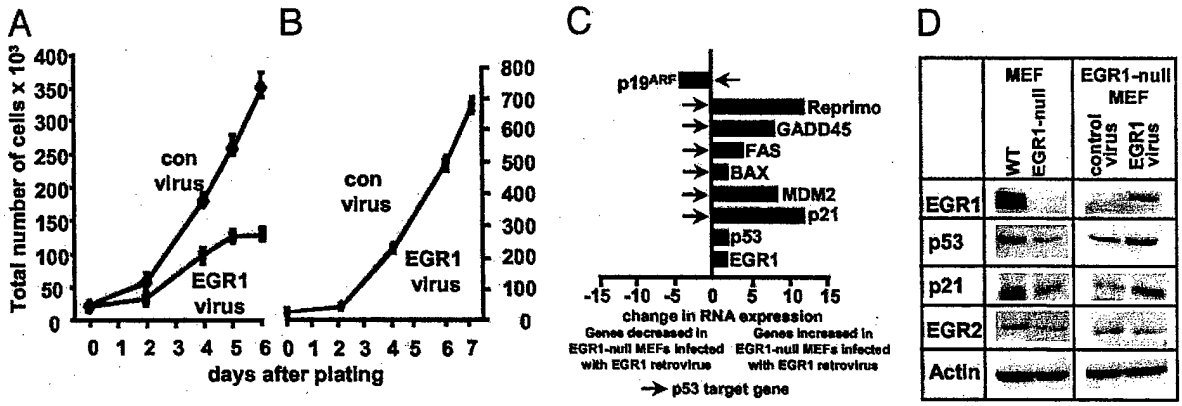

Fig. 3. Reexpression of EGR1 in EGR1-null MEFs restores replicative senescence. (*A*) MEFs from EGR1-null mice generated by Milbrandt and coworkers (15) were infected with an EGR1-expressing or an empty vector control virus and cultured in medium containing 75 μg/ml hygromycin B. After 2 wk of selection, the hygromycin-resistant colonies were counted and then seeded into six-well tissue culture plates at 20,000 cells per well. Cell numbers were determined by direct cell counting on days 2, 4, 5, and 6. Data are the average of three different wells of cells. Error bars indicate SDs (2σ) from the mean. (*B*) p53-null MEFs were infected with an EGR1-expressing virus or an empty vector control virus and were cultured in medium containing 75 μg/ml hygromycin B. After 2 wk of selection, the hygromycin-resistant colonies were counted and then seeded into six-well tissue culture plates at 20,000 cells per well. Cell numbers were determined by direct cell counting on days 2, 4, 6, and 7. Data are the average of three different wells of cells. Error bars indicate SDs (2σ) from the mean. (*C*) RNA expression levels were quantified by Q-PCR by using the 7900 Sequence Detection system from Applied Biosystems. Total RNA (0.5 μg) from EGR1-null MEFs infected with an EGR1-expressing virus or an empty vector control virus was reverse-transcribed into cDNA and amplified by using the SYBR-Green PCR-Master Mix and specific primers for each cDNA. Relative amounts of each gene were calculated by reference to standard curves and were then normalized to the relative amounts of GAPDH as detect in the same run. The fold change in RNA expression from EGR1-null MEFs infected with an EGR1-expressing virus as compared to EGR1-null MEFs infected with an empty vector control virus is shown. Arrows indicate known p53 target genes. (*D*) WT MEFs, EGR1-null MEFs, EGR1-null MEFs infected with an EGR1-expressing virus, or EGR1-null MEFs infected with an empty vector control virus were scored for EGR1 (≈80 kDa), p53, p21$^{inCip1/Waf1}$, and EGR2 (≈40 kDa) protein expression by Western analysis. Equivalent protein loading was confirmed by reexposing the same membranes to anti-β-actin Abs (≈48 kDa).

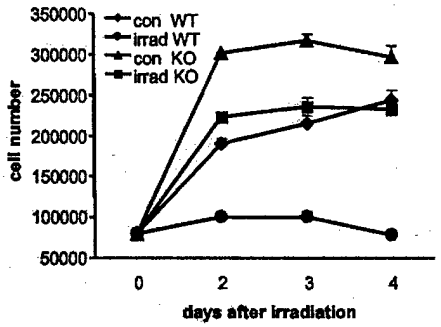

Fig. 4. DNA damage induced by irradiation leads to growth arrest of precrisis WT MEFs as compared to EGR1-null MEFs. Precrisis WT MEFs and EGR1-null MEFs were seeded into six-well tissue culture plates at 70,000 cells per well and were irradiated with 7.5 Gy. Cell numbers were determined by direct cell counting in duplicate on days 2, 3, and 4 after irradiation and 3 days after a subsequent reseeding (data not shown). Error bars indicate SDs (2σ) from the mean, and SDs with the symbol width are not visible. The experiment was replicated in triplicate with independent clones, which yielded very similar results.

suppressor gene. To test this hypothesis directly, immortalized p53-null MEFs (2) were infected with an EGR1-expressing retrovirus. Stably infected primary, early passage p53-null cells isolated in the presence of hygromycin-B, exhibited 2-fold increased EGR1 protein expression compared to cells infected with an empty vector control virus (data not shown). However, the p53-null MEFs that were productively infected with the EGR1-expressing retrovirus were not able to undergo senescence and showed the same growth curve as the parental p53-null MEFs infected with an empty control virus (Fig. 3*B*). These results indicate that WT p53 is an important downstream intermediate of EGR1-dependent senescence and growth suppression.

DNA Damage Does Not Arrest EGR1-Null MEFs at a Dosage That Arrests WT MEFs. To further confirm the observation that EGR1 is an upstream regulator of p53, precrisis MEFs (*Materials and Methods*) were seeded into six-well tissue culture plates and irradiated with 7.5 Gy to induce DNA damage. The subsequent proliferation of the irradiated cultures as well as nonirradiated controls was monitored by cell number counting in triplicate over the course of 5 days (Fig. 4). Inspection of the plates alone as well as cell counting revealed that WT cells exhibited a striking arrest in growth. These cultures remained very sparse with a high frequency of larger and flatter looking irregular cells. In contrast, the growth of irradiated Egr-1-null cells was brisk leading to a maximum density >3-fold than the irradiated WT cells (Fig. 4). Unirradiated WT cells also exhibited a rapid growth profile similar to irradiated Egr-1-null cells. In contrast, the growth of the irradiated WT cells is significantly reduced ($P < 0.01$) and the curve defines a broad plateau of little net growth over the 4-day postirradiation period. Moreover, when the cells are harvested and reseeded on day 5 at lower density, a common growth-stimulatory manipulation, irradiated EGR1-null cells resume growth whereas irradiated WT cells remain significantly arrested. Experiments with two independent MEF preparations lead to the same results (not shown). These results indicate that EGR1 is necessary to stimulate growth arrest after DNA damage and therefore further support that EGR1 is an upstream regulator of p53.

Inactivation of p53 Enhances Colony Formation in Postcrisis (High-Passage) WT MEFs Compared to Precrisis WT MEFs and High-Passage EGR1-Null MEFs. Our results suggest that enhanced unlimited growth of murine MEFs predominantly is due to the absence of intact EGR1 and its effect on the p53 tumor suppressor pathway. However, rare immortal WT MEFs can emerge. These cells invariably exhibit increased growth rate and ability to proliferate at low density because of mutations of the p53-MDM2-p19^{ARF} pathway (5, 27). However, if the role of p53 in promoting senescence in fact depends on EGR1 as indicated here, EGR1-null cells would be expected to be spared any mutations in p53 and to be protected from transformation.

WT MEFs became senescent after ≈17 passages, and postcrisis survivors became established as permanent cell lines (Fig. 1). To determine whether these cells had become transformed, colony formation assays were performed. Precrisis WT MEFs, postcrisis WT MEFs, or EGR1-null MEFs were plated at low density (600, 900, and 1,200 cells per plate) and were grown for 10 days. Staining and colony counting revealed that postcrisis WT MEFs had a greater ability to proliferate at low densities and formed 10-fold more colonies when compared to either precrisis or EGR1-null MEFs (Fig. 5*A*). Similarly, in replicate experiments (Fig. 5*A*), colonies were harvested with trypsin, and in parallel cultures the disaggregated cells were counted, which confirmed the large increase in proliferation by the postcrisis MEFs. The *t* tests indicated significantly increased proliferation for all replicate experiments: $P \geq 0.01$. To further assess transformation, 10 athymic mice were s.c. inoculated with postcrisis WT MEFs or EGR1-null MEFs. All mice inoculated with postcrisis cells developed tumors, whereas none of the 10 athymic mice inoculated s.c. with EGR1-null MEFs developed tumors. The difference is significant with $P < 0.0001$ (χ^2) (Fig. 5*B*). This experiment showed that postcrisis WT MEFs are highly tumorigenic in concordance with the colony formation results whereas EGR1-null cells, which had been in culture considerably longer than postcrisis WT cells, were entirely unable to develop tumors.

Given the importance of p53 in the regulation of cell growth, we analyzed the p53 status in the different cell types by performing immunoprecipitation assays with two Abs that recognize either WT or mutant p53 proteins (17, 18). As shown in Fig. 5*C*, postcrisis WT MEFs contained no detectable WT p53 protein whereas this protein was readily precipitated from high-passage EGR1-null MEFs. However, cell lysates of postcrisis WT MEFs contained a readily immunoprecipitated mutated form of p53 whereas mutant p53 could not be detected in lysates from high-passage EGR1-null MEFs precipitated with an anti-mutant p53 Ab (Fig. 5*D*).

Sequencing of a 764-bp region of the p53 gene (GenBank accession no. K01700) in precrisis MEFs, two different clones of postcrisis WT MEFs and high-passage EGR1-null MEFs starting at position 420 to position 1,183, which is homologous to a region in the human *p53* gene where most of the mutations occur, revealed point mutations at codon 211 in the postcrisis WT MEFs. One clone of postcrisis WT MEFs showed a C-to-G nucleotide exchange leading to serine-to-arginine change. The other clone of postcrisis WT MEFs showed a G-to-A nucleotide exchange leading to a serine-to-asparagine change. None of these mutations occurred in the precrisis WT MEFs or in the high-passage EGR1-null MEFs. Interestingly, the epitope of the Ab recognizing mutant p53 protein contains the codon 211 in which the mutation occurred (www.abcam.com/index.html?pageconfig=datasheet&intAbID=26), confirming the immunoprecipitation results (Fig. 5 *C* and *D*).

Thus, the results confirm that the transformed phenotype of postcrisis WT MEFs is exclusively associated with the absence of WT and presence of mutant p53. In contrast, in the precrisis state p53 appears to be normal. These results indicate that the reduced expression of functional p53 as observed here for the EGR1-null cells results in the unlimited and increased growth

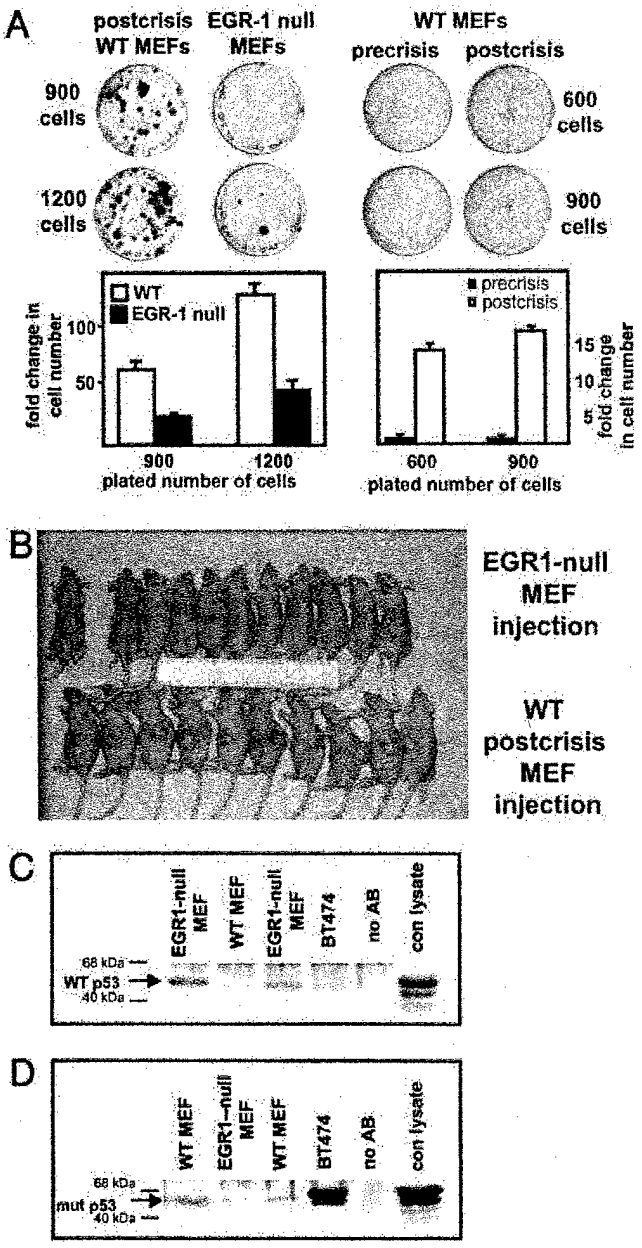

Fig. 5. Inactivation of p53 leads to a transformed phenotype of postcrisis (high-passage) WT MEFs as compared to precrisis WT MEFs and high-passage EGR1-null MEFs. (*A*) For colony formation, precrisis WT MEFs, postcrisis WT MEFs, or EGR1-null MEFs were counted and then seeded into 6-cm diameter tissue culture dishes at 600, 900, or 1,200 cells. After 8 days of incubation at 37°C, the colonies were stained with 2% crystal violet and cell numbers in parallel plates were determined by cell harvesting and direct cell counting. (*B*) Ten 6-wk-old female athymic mice were injected with postcrisis WT MEFs at passage 56, ten 6-wk-old female athymic mice were injected with EGR1-null MEFs at passage 62, and one mouse was kept without injection. Mice were monitored for 16 wk for tumor formation. (*C* and *D*) Lysates from postcrisis WT MEFs and EGR1-null MEFs were analyzed to determine the relative expression of WT and mutant p53 by immunoprecipitation using a conformation-dependent Ab (Pab 246) that recognizes WT (*C*) but not mutant p53 or an Ab (Pab 240) recognizing mutant p53 (*D*) but not WT p53 protein in its native form. Precipitates were used for Western analysis with a polyclonal p53 Ab. Lysates from BT474 human breast carcinoma cells, which are known to express mutated p53 (45), were used as a positive control for Pab 240. Mock-immunoprecipitated samples (Ab only omitted) and Western transfers that were not exposed to Ab were used as controls.

when compared to precrisis WT MEFs and indicates that Egr-1 is required for functional p53 expression and senescence.

Discussion

EGR1 Is a Growth Suppressor in Primary MEFs and Is Absolutely Required for Replicative Senescence. In most human tumors such as breast cancer, fibrosarcoma, and glioblastoma EGR1 is described to be a tumor suppressor gene (8–10). Paradoxically, higher levels of EGR1 were found in prostate cancer (28–31) and are thought to play a role in tumorigenesis. Therefore, it is very important to understand the growth regulation mechanism of EGR1. We investigated the role of EGR1 by use of contrasting genetic backgrounds of primary MEFs from WT and EGR1-null mice (15). Primary MEFs derived from WT mice as observed here exhibited many of the hallmarks attributed to replicative senescence (5, 32), including cessation of growth at low passage and increased expression of $p21^{Cip1/Waf1}$ followed by a marked decline in cell numbers and a deterioration of morphology. Cultures of WT MEFs that survive replicative senescence commonly exhibit mutations of p53 or, less frequently, genetic alterations of the major regulators of p53, $p19^{ARF}$, and MDM2 (33). Indeed, mutation of p53 itself or amplification of MDM2 or deletion of $p19^{ARF}$ all tend to inactivate p53-dependent regulation and promote transformation. The significance is shown by the fact that one of these changes occurs in 75% of cancers (33). At passage numbers considerably beyond the passage number characteristic of replicative senescence, we observed that MEFs lacking EGR1 are protected from mutations of p53. These cells retain the WT p53 sequence and therefore do not exhibit characteristics of transformation such as colony formation and tumorigenicity in contrast to cells containing mutant p53 that survive crisis are transformed (Fig. 5 *A* and *B*). The reduced expression of p53 in EGR1-null cells results in the unlimited and increased growth, which is not observed in precrisis WT MEFs. The observations presented here are in concordance with recent studies of Sherr and coworkers (5). In the view of Sherr and coworkers, senescence of WT MEFs is a phenotype of the *in vitro* (experimental tissue culture) environment. This environment promotes DNA damage that activates p53 thereby promoting the growth arrest and replicative senescence. Escape from senescence requires alterations of the p53-MDM2-$p19^{ARF}$ pathway, leading to transformation of the formerly euploid cells (5).

Consistent with a critical role for the p53-MDM2-$p19^{ARF}$ pathway, it was shown recently that the transcriptional repressors BMI-1 and TBX-2 inhibit senescence through down-regulation of $p19^{ARF}$ expression (34, 35). Furthermore, disruption of DMP-1, a positive regulator of $p19^{ARF}$ also leads to the bypass of senescence (36). Similarly $p19^{ARF}$-null MEFs are not able to undergo senescence (3), MEFs from $p16^{Ink4a}$-deficient mice do undergo senescence (37). These studies further illustrate the role of the p53-MDM2-$p19^{ARF}$ pathway in the regulation of replicative senescence. In addition, protein levels of $p21^{Cip1/Waf1}$, an important p53 target gene, are elevated in senescent human fibroblasts (38), and the $p21^{Cip1/Waf1}$ gene was identified in a screening for senescence-inducing genes (39). Nevertheless, the question of whether $p21^{Cip1/Waf1}$ is essential has yet to be unambiguously answered (40, 41).

EGR1 Is Required for the Function of p53. Taken together, our observations indicate that EGR1 is required for senescence by MEFs. These results suggest that EGR1 functions by activating the p53-MDM2-$p19^{ARF}$ pathway. Moreover, p53 is essential for the role of EGR1 in effecting senescence. Therefore, we propose that EGR1 represents a previously undescribed upstream gatekeeper of the p53 tumor suppressor pathway activity and, thereby, has an important impact on cell growth and cell-cycle progression. This function of EGR1 may apply to human tumors as well. EGR1 protein was found to be highly suppressed in 21 of

a series of 31 human gliomas when WT p53 was retained but nearly normally expressed in 10 cases with mutant p53, suggesting that expression of EGR1 is not required if p53 is inactivated (13).

The exact mechanism of EGR1-dependent regulation of p53 is unknown. However, it has been observed that EGR1 transactivates the p53 gene promoter (42, 43). Another potential regulatory interaction is suggested by Liu *et al.* (44) who identified a physical association between EGR1 and p53 *in vitro* and *in vivo*. It will be of interest, therefore, to examine whether these events are the basis of the gatekeeper function of EGR1 in cell cycle regulation.

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Insulin-like Growth Factor-II Regulates PTEN Expression in the Mammary Gland*

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The tumor suppressor PTEN is altered in many cancers, including breast cancer, but only a handful of factors are known to control its expression. PTEN plays a vital role in cell survival and proliferation by regulating Akt phosphorylation, a key component of the phosphatidylinositol 3 kinase (PI3K) pathway. Here we show that insulin-like growth factor-II (IGF-II), which signals through PI3K, regulates PTEN expression in the mammary gland. IGF-II injection into mouse mammary gland significantly increased PTEN expression. Transgenic IGF-II expression also increased mammary PTEN protein, leading to reductions in Akt phosphorylation, epithelial proliferation, and mammary morphogenesis. IGF-II induced PTEN promoter activity and protein levels and this involved the immediate early gene *egr-1*. Thus, we have identified a novel negative feedback loop within the PI3K pathway where IGF-II induces PTEN expression to modulate its physiologic effects.

PTEN[1] is emerging as the most frequently altered tumor suppressor gene other than p53 (1). PTEN is mutated in Cowden's syndrome, a condition of familial cancer predisposition, and is frequently altered in a variety of spontaneous cancers including breast cancers (2–4). The loss of even one PTEN allele in mice leads to a high incidence of tumors in a variety of tissues (3, 5). Breast cancer in humans is associated with a loss of heterozygosity or mutation of the PTEN gene, and decreased PTEN expression has been associated with invasive breast cancer and poor prognosis (2, 6, 7). The principal activity of PTEN is to dephosphorylate a phospholipid second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP3), produced by phosphatidylinositol 3 kinase (PI3K) (8, 9). PIP3 is the major activator of the cell survival kinase Akt (8, 9). Thus, negative regulation of the PI3K pathway by PTEN is critical, and the loss of PTEN function creates an environment conducive to tumorigenesis.

Despite the obvious importance of PTEN, only a handful of molecules are known to control its expression. Intracellular molecules reported to regulate PTEN transcription include p53 (10), peroxisome proliferator-activated receptor γ (11) and Egr-1 (12). These proteins induce PTEN promoter activity and putative binding sites for each have been identified in the PTEN promoter. Transforming growth factor β and progesterone have also been proposed to alter PTEN expression. Transforming growth factor β inhibited PTEN expression, but the nature of this regulation is unknown (13). Endometrial PTEN levels were higher during the secretory compared with the proliferative phase of the menstrual cycle implying an association with progesterone levels (14). Given the critical role of PTEN in the control of cell survival and proliferation, it stands to reason that extracellular factors such as hormones or growth factors should also influence PTEN expression.

Insulin-like growth factors (IGFs) are potent mitogens that impact development, are implicated as risk factors in breast cancer, and are overexpressed in human cancers (15, 16). IGF-I and IGF-II are produced by breast cancer cell lines (17, 18), and administration of IGFs to breast cancer cells promotes cell proliferation and inhibits apoptosis (19, 20). IGFs are known to mediate their cellular effects, at least in part, through the PI3K pathway (19). Here we demonstrate through biochemical, genetic, and molecular studies that IGF-II negatively regulates this pathway by increasing PTEN expression. Our results show that this PTEN regulation functions during mouse mammary development and that IGF-mediated PTEN regulation involves the immediate early gene *egr-1*.

MATERIALS AND METHODS

Mice, Tissue, and Serum Analyses—The generation of mouse mammary tumor virus (MMTV)-IGF-II mice has been reported (21). Mice were maintained following the guidelines of the Canadian Council on Animal Care. Whole mount, *in situ* hybridization, BrdUrd immunohistochemistry, serum progesterone, and 17-β-estradiol analyses were performed as previously described (22).

Mammary Tissue Manipulations—Administration of recombinant proteins to mammary glands involved injection of human IGF-II (rhIGF-II; Calbiochem, San Diego, CA) in phosphate-buffered saline with 0.1% bovine serum albumin or rhIGF-I (Calbiochem) and insulin (Sigma) in 10 mM acetic acid with 0.1% bovine serum albumin and 5 mM HCl. The 4th inguinal mammary glands were exposed surgically, and 10 μl containing 1 μg of rhIGF-II, 1 μg of rhIGF-I, and 10 μg of insulin or vehicle was injected. Elvax-40 pellets containing 300 ng of rhIGF-II or vehicle were generated (23) and respectively implanted into the 4th inguinal or contralateral mammary glands of 33-day-old wild type mice,

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[1] The abbreviations used are: PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3K, phosphatidylinositol 3 kinase; IGF, insulin-like growth factors; MMTV, mouse mammary tumor virus; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; MEF, mouse embryo fibroblasts.

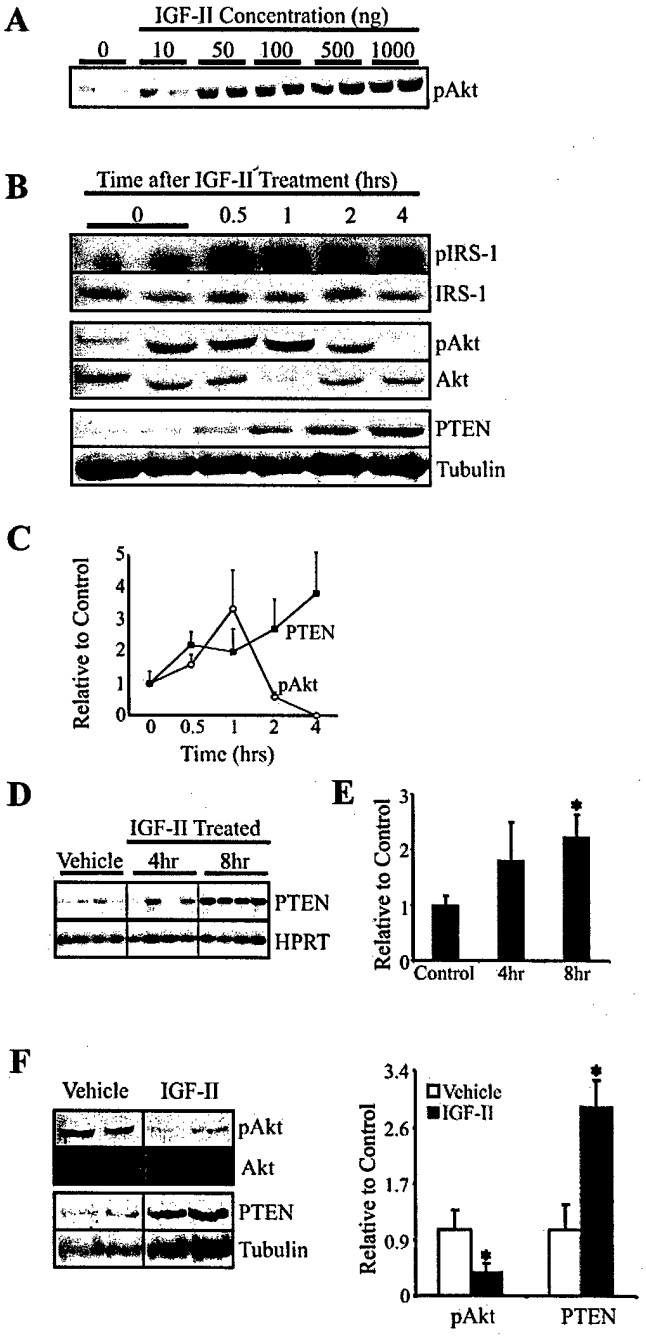

FIG. 1. **IGF-II induces mammary PTEN expression.** Western analysis of murine mammary (*A*) phosphorylated Akt 1 h after increasing doses of IGF-II. *B*, phospho-IRS-1, phospho-Akt, and PTEN at various times after injection of 100 ng of IGF-II; tubulin served as a loading control. *C*, quantification of the pAkt and PTEN levels in *panel B*. *D*, semi-quantitative reverse transcription-PCR and (*E*) real-time PCR of PTEN mRNA 4 or 8 h after IGF-II injection into mouse mammary tissue. *F*, levels of phosphorylated Akt and PTEN protein in mammary tissue taken from 49-day-old mice receiving a mini-osmotic pump containing vehicle (□) or recombinant IGF-II (■). *, $p < 0.05$.

and tissue analyzed 7 days later. For mammary transplants, the 4th inguinal fat pads of 21-day-old mice were cleared by removing the tissue between lymph node and nipple. A 2 × 2 mm piece of removed tissue was transplanted as described in the legend to Fig. 3. Whole mounts were analyzed 21 days later. Mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing 40 μg of rhIGF-II/100 μl of phosphate-buffered saline with 0.1% bovine serum albumin or vehicle (pump rate 0.25 μl/hr) were implanted in the abdominal cavity of 35-day-old wild type females and mammary tissue analyzed 14 days later.

Western Analysis—Protein isolation and Western blotting was performed as described previously (21). Antibodies were obtained from Cell

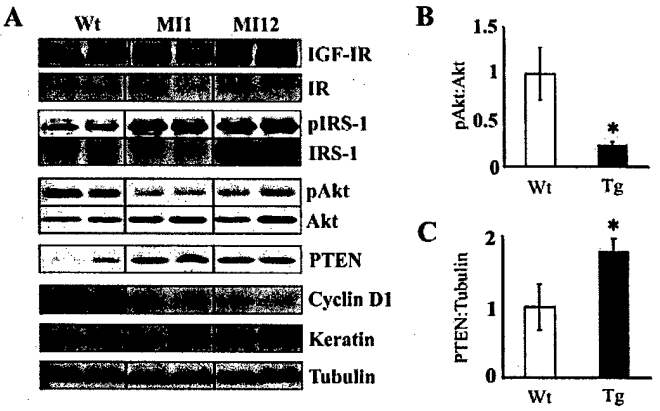

FIG. 2. **Transgenic IGF-II induces mammary PTEN protein.** *A*, Western analysis of phosphorylated IRS-1 and Akt as well as PTEN, cyclin D1, IGF-IR, insulin receptor, and keratin protein levels in mammary tissue from wild type and MMTV-IGF-II transgenic mice (*MI1* and *MI12*). Quantification of phosphorylated Akt (*B*) and PTEN Westerns (*C*). *, $p < 0.05$.

Signaling Technologies (Beverly, MA) except pIRS-1 (Medicorp, Montreal, Quebec, Canada), actin (Sigma), and cyclin D1, Egr-1, insulin receptor, IGF-IIR, α-tubulin, and keratin (Santa Cruz Biotechnology, Santa Cruz, CA).

Cells—Mouse embryo fibroblasts deficient for *egr-1* or *pten* were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (12).

Reverse Transcription-PCR and Real-time PCR—RNA was extracted from mammary tissue and reverse transcribed. Resulting cDNAs were amplified using a 56 °C annealing temperature and 23 cycles with primers for either PTEN (5′-ACAGACCTAGGCTACTGCTC-3′ and 5′-CTAGAAGCAAGACTTCCGTTC-3′), or the house-keeping gene hypoxanthine phosphoribosyl transferase (HPRT) (5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and 5′-GATTCAACTTGCGCTCATCTTAGGC-3′). PCR samples were electrophoresed, transferred to nylon membrane, and probed with the appropriate ^{32}P-labeled probe. Membranes were analyzed using a PhosphoImager and Imagequant software (Amersham Biosciences). Real-time PCR was performed as previously described (24) using a forward (5′-CCCAGTCAGAGGCGCTATGTATAT-3′) and reverse (5′-gttccgccactgaacattgg-3′) primer for PTEN and the probe (5′-CAGACCCGTGGCACTGCTGTTTCAC-3′).

Transfections and Luciferase Assays—Transfections and luciferase assays were performed as previously described with the following modifications (12). Following transfection with 500 ng of the reporter plasmid DNA, cells were placed in medium containing 0.5% serum and incubated at 37 °C for a further 24 h. Cells were then treated with IGF-II at a final concentration of 100 ng/ml for a period of 4 h and harvested at the appropriate time interval. All values were normalized using 100 ng CMV-β-galactosidase plasmid.

Statistics—All values are presented as mean ± S.E. Statistical significance was determined using the Student's *t* test, and values were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

IGF-II Injection Increases Mammary PTEN—We analyzed whether recombinant IGF-II administered in an acute or chronic regimen altered Akt and PTEN levels in mouse mammary tissue. Local injection into the gland increased phosphorylated Akt levels in a dose-dependent manner (Fig. 1*A*). As expected, IGF-II administration resulted in rapid and sustained phosphorylation of insulin receptor substrate 1 (IRS-1) (Fig. 1*B*). Despite this, Akt phosphorylation was only transient, peaking around one hour post-injection and declining to pre-injection levels an hour later (Fig. 1, *B* and *C*). Concomitant with phosphorylated Akt decline was an increase in PTEN protein (Fig. 1, *B* and *D*). IGF-II did not activate MAPK pathways as Erks or p38 were not phosphorylated at 1 h post-injection, whereas epidermal growth factor injection provided a positive control for Erk/p38 activation (data not shown). PTEN mRNA levels also rose over time (Fig. 1, *D* and *E*). Chronic exposure to elevated IGF-II was achieved through implanting

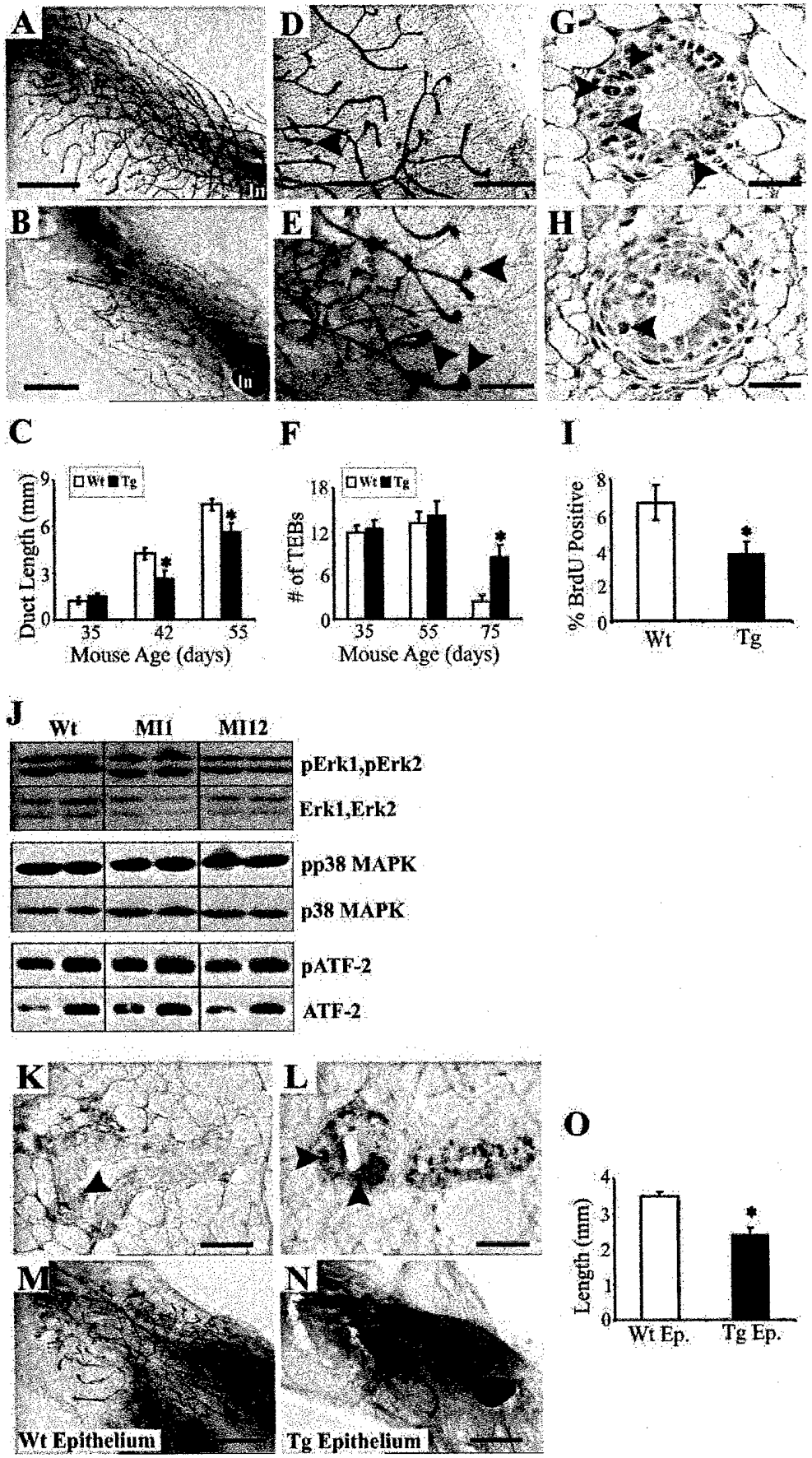

FIG. 3. **Reduced mammary ductal morphogenesis in MMTV-IGF-II mice.** Representative carmine-alum-stained wholemounts of wild type (*A*) and MMTV-IGF-II (*B*) transgenic mice at day 55 of development; *size bars*, 1 mm. *C*, quantification of ductal morphogenesis during mammary development. Representative wholemounts of wild type (*D*) and transgenic (*E*) 75-day-old mammary tissue. *Arrowheads* indicate TEBs; *size bars*, 400 μm. *F*, quantification of TEBs during mammary development. BrdUrd immunohistochemistry in wild type (*G*) and transgenic (*H*) tissue at day

mini-osmotic pumps containing IGF-II or vehicle. These pumps systemically released a constant amount of IGF-II over a 14-day period spanning 35–49 days of age. PTEN protein levels were significantly elevated in the mammary tissue of mice receiving this treatment. In parallel with chronic PTEN protein up-regulation, a significant reduction in the levels of phosphorylated Akt was observed (Fig. 1*F*). This demonstrates that IGF-II has the ability to influence molecules that both positively and negatively regulate PI3K signaling *in vivo*, through IRS-1 and PTEN, respectively.

Transgenic IGF-II Increases Mammary PTEN—To investigate the biological relevance of IGF-II-mediated PTEN induction in the mammary gland, we used MMTV-IGF-II mice that we have previously generated (21). We compared and found similar levels of type-I IGF (IGF-IR), insulin (Fig. 2*A*), and type-II IGF-II receptors (data not shown) in the wild type and transgenic mammary tissue. We then confirmed that IGF-IR was activated in transgenic tissue by assessing phosphorylation of IRS-1 in the developing mammary gland (Fig. 2*A*). Similar to our above findings with acute and chronic IGF-II administration, we found a significant reduction in phosphorylated Akt levels (Fig. 2, *A* and *B*), concomitant with a significant increase in PTEN protein levels (Fig. 2, *A* and *C*) in transgenic mammary tissue. Immunohistochemistry localized the reduced phosphorylated Akt and elevated PTEN protein to the transgenic mammary epithelium (data not shown).

IGF-II Inhibits Mammary Ductal Development—The PI3K/Akt/PTEN pathway is at the crux of cell survival, capable of influencing apoptosis, and more recently has been shown to regulate cell cycle progression (25, 26). Cyclin D1, a key component of cell cycle progression is a downstream effector of Akt (25). Diminished levels of phosphorylated Akt permit cyclin D1 degradation (26). Cyclin D1 levels were significantly lower in the IGF-II overexpressing mammary tissue in 55 day-old mice (Fig. 2*A*). At this age, mouse mammary tissue is normally undergoing intense proliferation associated with epithelial ductal morphogenesis. To determine the effects of IGF-II-induced PTEN and resulting reduction in PI3K signaling, we assessed mammary morphogenesis in MMTV-IGF-II mice. Mammary ductal morphogenesis was significantly retarded in transgenic mice (Fig. 3, *A–F*) as is evident by a reduction in epithelial duct length, number of ducts, and the presence of terminal end buds in 75-day-old mice. Terminal end buds normally disappear by this age, and their presence indicates that lengthening of the ducts is not yet complete (27). Consistent with this, mammary epithelial proliferation was significantly inhibited as measured by BrdUrd incorporation (Fig. 3, *G–I*). These phenotypes are opposite to those reported for conditional mammary PTEN knockout mice that have excessive ductal branching and mammary epithelial proliferation (28). This highlights the biological impact PTEN levels have in mammary morphogenesis.

Because pubertal mammary development is dependent on 17β-estradiol and progesterone (29), we measured the serum levels of these hormones and found no difference between transgenic and wild type mice (data not shown). This indicates that retarded mammary development did not arise from altered ovarian function in MMTV-IGF-II mice. We next determined whether chronic IGF-II exposure would phenocopy mammary ductal retardation. A slow-release recombinant IGF-II pellet was implanted in the developing mammary gland of wild type mice while the contralateral gland received a control pellet. The IGF-II pellet retarded mammary ductal length by an average of 26% (data not shown). These data show that ectopic IGF-II expression decreased epithelial proliferation and inhibited pubertal mammary morphogenesis.

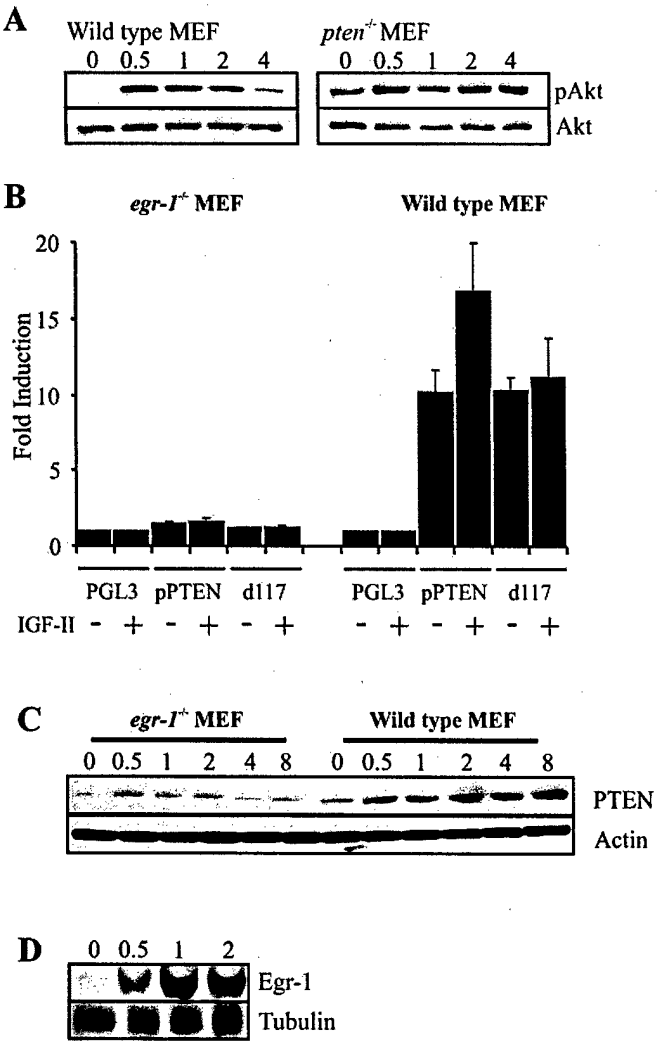

FIG. 4. **Egr-1 is required for IGF-II regulation of PTEN.** *A*, Western analysis of phospho-Akt in wild type and *pten*$^{-/-}$ MEFs at various times (h) after administration of 100 ng/ml of human recombinant IGF-II. *B*, PTEN promoter activity following IGF-II treatment in wild type and *egr-1*$^{-/-}$ MEFs. *PGL3*, control vector; *pPTEN*, 2kb of PTEN promoter; *d177*, PTEN promoter mutated at the three putative Egr-1 binding sites. *C*, Western blot analysis of PTEN protein in wild type MEFs and *egr-1*$^{-/-}$ MEFs at indicated times (h) following IGF-II administration. Representative Western blots of Egr-1 protein levels at indicated times (h) upon IGF-II administration in wild type mammary tissue (*D*).

Other well established signaling pathways linked to cell proliferation include Erk1/Erk2, p38 MAPK, and JNK/SAPK. However, no significant differences were found in the levels of phosphorylated Erk1/Erk2 or p38 MAPK in transgenic mammary tissue at day 55 (Fig. 3*J*). We assessed ATF-2, which is downstream of JNK/SAPK, and observed that ATF-2 phosphorylation was also unaltered (Fig. 3*J*). Thus, we ruled out the involvement of these potential pathways in mediating the inhibitory effect of IGF-II on epithelial proliferation.

55 of development; *size bars*, 75 μm. *Arrowheads* denote BrdUrd-positive epithelial cells. *I*, quantification of BrdUrd-positive cells. *J*, Western analysis of phosphorylated Erks, p38 MAPK, and ATF-2 in wild type and transgenic mammary tissue. *In situ* hybridization for IGF-II in wild type (*K*) and transgenic (*L*) mammary tissue. *Arrowheads* denote IGF-II positive cells. Representative wholemounts of wild type (*M*) epithelium transplanted into a cleared transgenic fat pad and transgenic (*N*) epithelium transplanted into a wild type fat pad. *O*, quantification of transplant growth. *Wt*, wild type; *Tg*, transgenic; *Ep.*, epithelium.

IGF-II Effect Is Epithelial Cell Autonomous—Both epithelial and stromal factors influence ductal development (23, 30). We found that transgenic mammary IGF-II expression was exclusive to epithelial cells (Fig. 3, *K* and *L*). To determine whether epithelial IGF-II was sufficient to delay ductal progression, reciprocal mammary transplants were performed between wild type and transgenic mice. Cleared mammary fat pads were generated in both wild type and transgenic mice by severing the epithelial ductal trees prior to puberty. Transgenic mammary epithelium was implanted into wild type cleared fat pads (Fig. 3*N*), whereas wild type tissue was implanted into cleared transgenic fat pads (Fig. 3*M*). Epithelial ducts emanating from the transgenic transplants were significantly shorter than those from wild type transplants (Fig. 3*O*). Thus, this phenotype was epithelial cell autonomous; epithelial IGF-II rather than the host environment (stromal and endocrine factors) was responsible for the retarded mammary development. Because human breast cancer originates in epithelial cells, the existence of an IGF-II/PTEN link in epithelial cells and its relation to mammary growth is particularly relevant.

IGF-II Regulates PTEN Transcription via Egr-1—We first tested the requirement of PTEN in IGF-II mediated Akt inactivation. Akt phosphorylation was sustained upon IGF-II treatment in *pten*$^{-/-}$ MEFs in relation to the wild type MEFs. Additionally, the basal level of Akt activation was substantially higher in *pten*$^{-/-}$ MEFs (Fig. 4*A*). To determine whether IGF-II regulates PTEN transcription, we used a PTEN-luciferase construct, pPTEN. MEFs transfected with this construct showed significant induction of luciferase activity after IGF-II treatment compared with a control PGL3 vector (Fig. 4*B*), demonstrating that IGF-II is able to induce PTEN promoter activity. A similar increase in PTEN promoter activity was also observed in NIH3T3 cells (data not shown). The IGF axis is able to induce the transcription factor *egr-1* in embryonic and cardiac fibroblasts (31, 32), and we have recently shown that *egr-1* directly activates PTEN during irradiation-induced signaling to a similar magnitude as observed above (12). Therefore, we investigated the requirement of *egr-1* for IGF-II regulation of PTEN transcription. MEFs transfected with PTEN-luciferase constructs lacking the three putative *egr-1* binding sites (d117), but retaining the p53 binding site, failed to show luciferase induction following IGF-II treatment (Fig. 4*B*). Furthermore, *egr-1*$^{-/-}$ MEFs containing intact PTEN promoter constructs also showed no induction of luciferase activity when treated with IGF-II (Fig. 4*B*). The higher basal reporter activity in wild type cells may be reflective of its higher p53 levels compared with the *egr-1*$^{-/-}$ cells. In addition, IGF-II administration resulted in elevated PTEN protein levels in wild type MEFs but not *egr-1* null MEFs (Fig. 4*C*). Furthermore, we found that IGF-II injection into the mouse mammary gland induced Egr-1 protein expression *in vivo* (Fig. 4*D*). At present, the receptors that mediate the IGF-II effect remain to be elucidated.

In this study we demonstrate that the growth factor IGF-II induces expression of the tumor suppressor gene PTEN. PTEN is the major negative regulator of PI3K signaling, the very pathway used by IGFs to transmit their growth-stimulatory signal. This represents the first example of a negative feedback loop in IGF signaling that operates through PTEN to control proliferation. The biological consequences of this feedback are illustrated by experimentally increasing IGF-II levels, which lead to reduced proliferation and delayed mammary development. Regulation of PTEN occurs at the transcriptional level and the immediate early gene *egr-1* is a necessary component of this loop. Similar feedback loops have been demonstrated in other signaling pathways including signaling from the insulin receptor (33, 34).

The canonical IGF-II pathway is mitogenic and implicated in mammary carcinogenesis. Our study demonstrates that through up-regulation of PTEN, IGF-II exerts a hypomorphic effect during mammary gland development. This effect bears a striking similarity to the MMTV-*neu* mouse, a widely used model of mammary tumorigenesis that also has hypomorphic mammary glands (35). Our findings provide mechanistic insights into the complexity of oncogene action, whereby a growth factor can restrain its own mitogenic action by up-regulating a key tumor suppressor. Loss of this negative feedback loop may release the oncogene's cancer promoting ability, allowing its proliferative effect to dominate.

We have found that the immediate early gene *egr-1* is vital to the induction of PTEN by IGF-II. Studies have shown that *egr-1* is an integral player in the IGF axis. It induces IGF-II promoter expression in HepG2 cells in response to hypoxia (36). Also, stimulation of IGF signaling induces *egr-1* expression, an effect that may be dependent on IRS-1 (31, 32). Thus, *egr-1* is both upstream and downstream of IGF-II signaling. It is conceivable that another level of regulation exists within the IGF-II/PTEN negative feedback loop wherein IGF-II signals through *egr-1* to induce PTEN, but *egr-1* also leads to IGF-II induction. Such multiple negative and positive regulatory loops likely operate within all cells to temper the actions of external growth stimuli.

We have previously examined the PI3K/Akt/PTEN pathway at a different stage of mammary physiology in MMTV-IGF-II mice, namely post-lactation involution. We found that, unlike during mammary development, IGF-II did not influence PTEN expression during mammary involution (21). This finding is not surprising because the two stages have diametrically opposed cell fates and are structurally, hormonally, and functionally distinct. The developing mammary gland is primarily composed of ductal epithelium proliferating under the influence of estrogen and progesterone, whereas involuting mammary gland is lobulo-alveolar epithelium undergoing apoptosis initiated by prolactin withdrawal (37, 38). The marked differences at these stages likely account for the disparate effects of IGF-II on PTEN.

Egr-1 and PTEN, the components of our proposed negative feedback loop of IGF-II signaling, have been individually implicated in breast cancer (2, 6, 7, 39). It is possible that alterations in *egr-1* and/or PTEN break the negative feedback loop, allowing the proliferative effects of IGF-II to dominate. It is important to note that loss of PTEN function in breast cancer is predominantly through loss of expression and not mutation. Our findings describe a potentially important PTEN regulatory pathway involving the breast cancer mitogen, IGF-II.

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